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C.-E. A. WINSLOW

It is characteristic of Science and Progress that they continually open new fields to our curiosity.—PASTEUR.

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SALT EFFECTS IN BACTERIAL GROWTH[1]

I. PRELIMINARY PAPER

GEORGE E. HOLM AND JAMES M. SHERMAN

From the Research Laboratories of the Dairy Division, United States Department of Agriculture, Washington, D. C.

Received for publication February 28, 1921

The Hofmeister series shows the effects of ions of neutral salts upon the coagulation of colloids and upon the swelling and other physical properties of proteins. Our knowledge of these ion effects in solution has been greatly extended by Freundlich and his students. They noted that the ions could be arranged in a definite order with respect to their effects upon compressibility, surface tension, solubility, viscosity, absorption, ratio of reaction, etc. Freundlich seems to favor the hydration theory of salts as an explanation of this neutral salt action, and since the properties affected are so closely related and bound up with one another, and the ions so consistent in their order of effect, he calls these effects "lyotropic" effects. The lyotropic explanation does not lay claim to being a full explanation of neutral salt action, but it does lay claim to correctness in that it systematically treats complicated phenomena.

In most cases the influence of the anion far outweighs that of the cation and the order of anion effects usually reads as follows, $F > SO_4 > PO_4 > Cl \geq NO_3 > Br > I > CNS$; while the order of arrangement for the cation is usually $Ca > Sr > Mg > Cs > Rb > K > Na > Li$. The same sequence is obtained in the widely differing changes mentioned above and does not seem to follow any recognizable order with respect to valency, atomic weight, etc. Certain reagents may promote or hinder the salt effects as compared with those in pure solution. In some cases the

[1] Published with the permission of the Secretary of Agriculture.

order of effects may be reversed when changes take place in acid or in alkaline solutions, but the sequence usually remains the same.

The lyotropic effects of salts upon compressibility, surface tension, solubility, etc., of organic and inorganic substances in solution is not great in most cases. It is in the field of colloid chemistry that these effects attain a magnitude of great significance. A review of the literature covering this field is out of the question here, but a few citations from the biochemical field will serve to show the reasons for extending the work to the field of bacteriology.

In view of the recent and extensive investigations of Loeb (1918–1921) upon the physical and chemical properties of proteins it will probably be necessary to modify certain conceptions now held concerning the relative magnitude of various ionic effects in protein solutions. As to whether the Hofmeister series of ions will be shown to be entirely a delusion, as is believed by Loeb, we do not care to express an opinion, and it is not the purpose of the present paper to take sides on that controversial question.

Whatever may be the status of ion effects in protein chemistry, specific ionic effects in biological phenomena have been well established by the work of Loeb and many others. It is our purpose to study systematically salt effects, especially the qualitative and quantitative relationships of radicals (anions and cations), as related to bacterial growth, and to correlate these findings with other effects which have been noted in pure chemistry as well as in biology.

Closely related to the phenomena of hydration and coagulation is that of permeability and diffusion. On the basis of the view of Bechhold and Ziegler (1919) that membranes do not act like sieves, but as though they were a network of arranged ions, it is easy to conceive of enormous salt effects upon permeability, both by influence upon ions which are to diffuse and by effects upon ions forming the membrane network.

Bacteria perhaps represent matter in a state as near the state of colloids and also as near the state of living protoplasm as

any organism does. The effects of salts should therefore not only be very marked but might reach magnitudes that ought to be taken into account in the culture of bacteria.

Brooks (1919) found that NaCl and KCl in concentrations of 0.15 to 0.20M increased the rate of respiration of *B. subtilis*, while in higher concentrations they decreased the rate. $CaCl_2$ increased the rate in a concentration of 0.05M and decreased the rate in higher concentrations. Dealing with the respiration of *Aspergillus niger*, Gustafson (1919) likewise found a stimulation by NaCl in concentrations of 0.25 to 0.5M and by 0.5M $CaCl_2$.

The work of Winslow and Falk (1919) shows that NaCl and $CaCl_2$ both increase the mortality of *Bact. coli* in water. In the case of NaCl 5 isotonic was distinctly lethal, while in the case of $CaCl_2$ 0.1 isotonic was injurious.

Greaves (1916) found the toxicity of anions as measured by ammonification in soils to be in the following order: $Cl > NO_3 > SO_4 > CO_3$. He also noted that the toxicity of some salts increases more rapidly with increased concentration than does that of others. This action he ascribes to the physiological factor of the organism rather than to the osmotic pressure or salt action of the solution.

The influence of alkaline salts upon phagocytosis was found by Radsma (1920) to depend mainly upon the anions but also somewhat upon the cations. Radsma explains the effect as surface action and considers it an indication of colloidal chemical structure of protein substances at the surface.

Mathews (1906) pointed out that the action of salts upon the protoplasmic system is due chiefly to the ions of the salts and he considers the physiological action dependent upon the available potential energy.

Whether or not the salt action upon bacteria is due to the available potential energy of the ions we shall not attempt to decide. We merely wish to point out in this paper that salts do affect bacterial growth much in the same manner as they affect chemical reactions, coagulation, permeability, etc., that

this effect is modified by the hydrogen ion concentration of the medium, and that such effects are probably great enough to be given consideration in bacterial culture.

EXPERIMENTAL

The organism used was *Bact. coli*, and the basic medium chosen was a 1 per cent pepton solution to which was added the crystalline salts in amounts necessary to give the desired concentrations. The media were autoclaved and filtered in case of the formation of a slight precipitate. At this point the pH was adjusted if necessary with HCl or NaOH, and the media tubed and sterilized. These tubes containing 10 cc. were in each case inoculated with a loopful of a young culture and incubated at 37°C.

There was some question as what should constitute a measure of bacterial growth and what factor would be constant enough for comparative purposes. The reduction of methylene blue was first tried. In this case a layer of paraffin oil was used to prevent oxidation by the air. Although it worked quite satisfactorily, it was found that reoxidation occurred in the cases where bacterial action was slow, and thus, instead of giving a sharp end point, really increased the time for reduction. The rapidly growing cultures gave a sharp end point. It was noticed, however, that a slight turbidity was apparent in most cases before reduction could be detected. It was decided, therefore, to use the first sign of turbidity as an indication of the rapidity of bacterial growth.

A few trial experiments indicated that the two methods of detecting growth checked very well, except for the fact that turbidity was first detected and proved a sharper measure than reduction. The first sign of turbidity when the tubes were held against a strong artificial light was therefore used to measure rapidity of growth. This method was further verified by growing the same organism in a medium of 1 per cent pepton containing 1 per cent lactose and adjusted to a pH of 7.0. The production of acidity paralleled the results obtained by reduction and visible turbidity.

Inasmuch as the anionic effects seem to be predominant in chemical reactions it was decided to try the effects of salts having a common cation (sodium). The salt concentration chosen to be used was 0.20 molar, which was low enough to give the ionic effects and not too pronounced osmotic effects of the salts. To eliminate as nearly as possible H-ion effects the pH was adjusted to 7.0, colorimetrically, before final sterilization.

TABLE 1

Showing the effect of various sodium salts upon the rate of growth of Bact. coli

MEDIUM	pH	TIME REQUIRED TO SHOW TURBIDITY
		hours
1 per cent pepton	7.2	4½
1 per cent pepton 0.20 M NaCl	7.3	3½
1 per cent pepton 0.20 M NaI	7.3	3½
1 per cent pepton 0.20 M $NaNO_3$	7.3	3½
1 per cent pepton 0.20 M Na_2SO_4	7.0	4
1 per cent pepton* 0.20 M Na H PO_4	7.3	4½
1 per cent pepton† 0.20 M Na lactate	7.0	4½
1 per cent pepton 0.20 M Na oxalate	7.0	9½
1 per cent pepton 0.20 M Na acetate	7.0	10½
1 per cent pepton 0.20 M Na citrate	7.3	10½
1 per cent pepton 0.20 M Na fluoride	7.4	48

* Mono-and di-sodium phosphate were mixed in proper proportions to give a pH of approximately 7.0.

† The sodium lactate used was prepared by adding NaOH to lactic acid until a pH of 7.0 was reached.

Table 1 shows the effects of various sodium salts upon the growth of *Bact. coli*. The table indicates that the Cl, I, NO_3, SO_4, PO_4, and lactate ions accelerate growth of *Bact. coli*, while the other ions tried inhibit to a greater or less extent. Using the Cl, I, SO_4, and lactate ions in the same concentrations, the series was repeated with the following results:

Medium	*Time hours*
1 per cent pepton	4½
1 per cent pepton 0.20 M NaCl	3½
1 per cent pepton 0.20 M NaI	3½
1 per cent pepton 0.20 M Na lactate	3½
1 per cent pepton 0.20 M Na_2SO_4	4½

Table 1 indicates that there is a marked effect of salts upon the growth of *Bact. coli*, and it would seem that it is largely due to the anion.

To find out to what extent the cation affects such growth the effect of the following salts were tried: KCl, NaCl, NH_4Cl, $MgCl_2$, $CaCl_2$, and $FeCl_3$. Table 2 gives the effects of 0.20 molar concentrations of these salts upon growth. Table 2 seems to indicate that there is little difference between the effects of the Na, K, and NH_4 ions. Since in the case of $MgCl_2$ we have twice the concentration of Cl ions which we have in the former, a true comparison cannot be made if the anionic effects predominate. To make our experiments comparable we compared growth in a 0.20 molar NaCl pepton medium with growth in

TABLE 2

Showing the effect of various cations upon the rate of growth of Bact. coli

MEDIUM	pH	TIME REQUIRED TO SHOW TURBIDITY
		hours
1 per cent pepton	Approx. 7.0	5
1 per cent pepton 0.20 M NaCl	" 7.0	$3\frac{1}{2}$
1 per cent pepton 0.20 M KCl	" 7.0	$3\frac{3}{4}$
1 per cent pepton 0.20 M NH_4Cl	" 7.0	$3\frac{3}{4}$
1 per cent pepton 0.20 M $MgCl_2$	" 7.0	8
1 per cent pepton 0.20 M $CaCl_2$	" 7.0	120
1 per cent pepton 0.20 M $FeCl_3$	" 7.0	No growth

0.10 molar $MgCl_2$ pepton medium. The effects of 0.40 molar NaCl and 0.20 molar $MgCl_2$ were also tried.

The results are shown in table 3. $MgCl_2$ and NaCl, therefore, in concentrations where the number of Cl ions is the same, are comparable in effect. That there is a cation effect in greater concentrations, however, is shown by the fact that the time for 0.20 molar $MgCl_2$ is 12 hours, while that for 0.40 molar NaCl is but $4\frac{3}{4}$. Doubling the NaCl concentration changes the time rate very little, while doubling the $MgCl_2$ concentration more than triples the time. This is strong evidence that there is a cation effect, though it may not be so marked as the anion effects. $CaCl_2$ has a much stronger inhibiting effect while $FeCl_3$ entirely inhibited growth in the concentration used.

Our results so far have been obtained upon media adjusted to a pH of approximately 7.0. Since we know that the H-ion concentration materially affects growth of bacteria, it is of both interest and value to know to what extent the salts modify the time element at pH values on either side of neutrality. Table 4 shows these effects with the salts given and at the H-ion concentrations stated. The results indicate that the different salts

TABLE 3

Showing the effects of various concentrations of NaCl and of $MgCl_2$ upon the rate of growth of Bact. coli

MEDIUM	pH	TIME REQUIRED TO SHOW TURBIDITY
		hours
1 per cent pepton 0.20 M NaCl	7.0	3¼
1 per cent pepton 0.40 M NaCl	7.0	4¾
1 per cent pepton 0.10 M $MgCl_2$	7.0	3¼
1 per cent pepton 0.20 M $MgCl_2$	7.0	12

TABLE 4

Showing the effects of various salts upon the growth of Bact. coli at different H-ion concentrations

MEDIUM	TIME OF VISIBLE GROWTH AT pH VALUES OF				
	5.2	6.2	7.6	8.2	9.2
	hours	hours	hours	hours	hours
1 per cent pepton	17½	5¾	6½	8	32
1 per cent pepton 0.20 M NaCl	5½	4½	3½	3	14
1 per cent pepton 0.20 M Na_2SO_4	6½	4½	4¼	3½	
1 per cent pepton 0.20 M Na citrate	26	8½	7½	22	

have marked changes of effect with changes in pH. In general we may say that NaCl and Na_2SO_4 widen the optimum range of growth, while Na citrate narrows this pH range.

DISCUSSION

The data presented show in a general way some correlation between the so-called lyotropic series and the order of effect upon the growth of *Bact. coli*. There are, however, ions which

are exceptions and which, in concentrations thus far tried, have proved highly retarding in their action. These ions are the sulphocyanate and fluoride. Whether in lower concentrations they might not prove but slightly retarding or even beneficial to growth remains to be ascertained. The position of the SO_4 radical with regard to effect upon bacterial growth is also somewhat at variance with its usual position in the lyotropic series. Instead of being found opposite the iodine end of the series it is found next to the Cl and I radicals.

While these are deviations from the usual order, it is not surprising since in many of the phenomena in biochemistry the lyotropic order does not strictly compare with the usual order as determined by effects upon surface tension, viscosity, etc., especially at different concentrations and temperatures. We must also remember that here we are dealing with an added factor which is not present with proteins in solution or with colloids in general; that is, the life of an organism. Since this is our measure of effects it must be taken into account. We do not know what properties affect viability most, and consequently we have no means of knowing what mechanism causes retarding and inhibitory effects. There seems to be, as might be expected, an order of specificity which must be taken into account.

The general order of the lyotropic series, however, holds at pH 7.0 Cl and I are found at one end of the series aiding or accelerating action, while the citrate, acetate, and oxalate at the other end retard growth.

Considering the effect of cations, we have a close analogy between action here and action of salts in the animal body. As might be expected, there is little difference between the Na, K, and NH_4 ions. With Mg the action begins to manifest itself, although it is greatly modified by the anion effect. The calcium ion produces its characteristic strong effect.

It is in media of different H-ion concentrations that these effects become significant. In the region of optimum growth the influences are not exceedingly marked, but as we near the H-ion concentrations which mark the limits for growth of *Bact. coli*

the differences in the rate of growth are greatly increased. In other words the H-ion range for optimum growth is widened or narrowed as shown in the table given.

This factor becomes of practical value in adjusting media for optimum bacterial growth. The figures show that certain ions are of value in pepton media for accelerating the growth of *Bact. coli* and also for widening the range for optimum growth, while certain other ions narrow the H-ion range and decrease the rate of growth. This would perhaps explain the findings of Cohen and Clark (1919) that culture media adjusted with HCl had a higher limit of growth on the acid side than media adjusted with acetic acid.

SUMMARY.

It has been shown that the growth of *Bact. coli* in 1 per cent pepton medium is accelerated or retarded by different salts in low molecular concentrations.

The salt effects at various H-ion concentrations vary greatly. Those salts which accelerate growth seem to widen the H-ion range for optimum growth, while those which retard growth seem to narrow the limits for optimum activity.

Cations and anions are both effective.

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SUGGESTIONS CONCERNING A RATIONAL BASIS FOR THE CLASSIFICATION OF THE ANAEROBIC BACTERIA[1]

STUDIES IN PATHOGENIC ANAEROBES. IV

I. PRELIMINARY PAPER

HILDA HEMPL HELLER

From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco

Received for publication March 1, 1921

During the past three or four years I have made a study of a carefully controlled series of certain groups of anaerobic strains secured from pathological material. Attention was directed almost entirely to such strains as were found to be capable of penetrating living guinea-pig muscle in doses of 1 cc. or less of young ground beef-heart culture. *B. Welchii* was not considered, and this organism is not included in the following list. Though I was forced for want of time to neglect non-pathogenic forms, such organisms are so frequently encountered in a study of pathological material that one who has collected anaerobes of one group must necessarily observe those of other sorts and learn something of their ways. The pathogenic tissue-invading strains included in my collection are 80 in number: 23 from human wound infections, 32 from cases of so-called "blackleg" of cattle, 10 from cases of braxy and of blackleg of sheep, and 15 from other animals. The collection includes 30 odd strains of tetanus and other proteolytic organisms of various sorts.

[1] This work and that described in the following papers was commenced during the author's tenure of the Alice Freeman Palmer Fellowship of Wellesley College.

All strains were carefully isolated and the cultures were continually observed in order to detect contaminations.[2]

The samples collected, though they do not exhaust the pathological material from anaerobic infections, are very widely representative, and the collection of much more material and the isolation from it of many more pathogenic strains would be an exceedingly arduous task. It must also be borne in mind that in greatly increasing the number of strains under observation one must necessarily relax the vigilance with which he criticises the purity of those strains which are studied. The examination of any considerable number of anaerobes is a comparatively new task, and any proposal for classification which is made at the present period is bound to be a temporary one. The time is unquestionably not ripe for an elaborate study of several hundred strains of any particular type of anaerobe because the material for such a study has never been collected, and such a collection would represent several years' work and a considerable outlay for experimental animals. But the cultures that I have been able to isolate during the past few years have furnished so much material for investigation, and the information gained from them has so radically altered my attitude toward the anaerobic group, that I feel that the time has arrived to state my results, to organize them as consistently as may be, and to propose a system for their classification. In other words I feel that the status of the classification of the anaerobes is today so chaotic and unsatisfactory that a pioneer effort at a logical grouping according to our present knowledge is very much needed. If we consider the fact that the investigated material, when compared with the vast amount of uninvestigated material, is exceedingly scanty, we shall not expect such a classification to be final. We have today, however, a fairly definite conception of the pathogenic anaerobes, and by analyzing the

[2] An account of the affinities of the animal strains studied will be found in part in The Journal of Infectious Diseases for November, 1920, Vol. 27, and in full in the Collected Reprints of the Hooper Foundation for 1921, Vol. VI, under the title "Etiology of Acute Gangrenous Infections of Animals." This paper contains a description of the methods employed in the isolation of my cultures.

groups thus studied we shall be enabled so to orient our classification of the whole anaerobic group that some conception of its size and general relationships will be presented for the use of future investigators.

The uncertainties prevailing in the classification of the anaerobes, apparent to anyone who has tried to identify an isolated anaerobic strain, become more glaring as one proceeds to study several strains that were handed to him under the same name, but which display great differences in their behavior. Later, on continuous study of anaerobes of various types, and of the literature of anaerobic infections, the worker comes to the realization that identifications by means of descriptions found in the existing literature can at best be only tentative and approximate, and that the majority of those type strains on which were based the descriptions to which we are compelled to refer for priority are now lost or badly contaminated. Thus a large number of the older descriptions are potentially invalidated, or orphaned, so to speak, and cannot, today, be used for any definite systematic purpose, and the names proposed in those descriptions are now *nomina nuda*. This situation must be deliberately faced. What we need is an elastic, adaptable system of classification in which the old descriptions can find a place as well as the new: a system consistent, also, with the Rules of Botanical Nomenclature, whose adoption has been proposed by the Committee of the Society of American Bacteriologists.

FORMER CLASSIFICATIONS

It is necessary to consider what have been the methods of classifying our group that have been proposed by other workers.

Zopf defined the genus *Bacillus* as including: "Cocci and rods with spores" and the genus *Clostridium* as: "Like *Bacillus* but spores in spindle-shaped elements."

Kruse (1896, pp. 67 and 185) included in his family of *Bacillaceae* three groups which comprise the anaerobes. They are:

6. The malignant oedema group: large spore-bearing anaerobic bacilli. Saprophytic or parasitic. Colonies on agar usually stellate.

No change in form of mother cell on spore formation. Less easily stained by Gram method than preceding group (anthrax group). Usually liquefy gelatin and produce foul odors.

7. The symptomatic anthrax and butyric acid group. Large bacilli that swell to form clostridia on sporulation, saprophytic and parasitic, mostly anaerobes.

8. Tetanus group. Fairly large bacilli with drumstick (Knöpfchen) spores. Mostly anaerobic parasites and saprophytes.

Migula (1900) included in his family *Bacteriaceae* the peritrichially flagellate rods in one enormous genus, *Bacillus*; the non-flagellate rods in another, *Bacterium*.

Fischer (1903) in his second classification divided the *Bacillaceae* into: Sporulating and non-sporulating rods, sporangia unchanged in shape, *Bacillieae*; and spore-bearing rods modified in shape: spindle-shaped, *Clostridieae*, and drumstick shaped, *Plectridieae*.

Lehmann and Neumann (1904) divide the *Bacteriaceae* into the genera: "*Bacterium*, without endogenous spores, rods usually under 0.8 to 1μ in diameter," and "*Bacillus*, with endogenous spores, rods often more than 1μ in diameter."

Orla-Jensen (1909) in his comprehensive reorganization of systematic bacteriology according to the chemical behavior of the organisms, proposed for the higher bacteria the order *Peritrichinae*, to include rods and spherical forms which show a marked tendency to split carbohydrates and amino-acids. In this order he proposed four families, two aerobic and two anaerobic, two producing acid and two producing alkali. The anaerobic families he called *Butyribacteriaceae* and *Putribacteriaceae*. He would place with these, I presume, the anaerobic cocci, and spore-formation is evidently not a requisite criterion for admission to the anaerobic groups. In the *Butyribacteriaceae* he proposed three genera: *Butyribacillus* (to include *B. Welchii* and *B. Chauvoei*), *Pectobacillus*, and *Cellulobacillus*. In the *Putribacteriaceae* he proposed the genera *Putribacillus* and *Botulobacillus*, the latter to include *B. botulinus* and *B. tetani* on account of their toxin production.

The Committee of the Society of American Bacteriologists (1920, classification first formulated in 1917) places in the family (no. VII) *Bacillaceae*, sporulating rods, two divisions which are ranked as genera. These are: 1. The genus "*Bacillus* Cohn 1872 Aerobic forms. Mostly saprophytes. Liquefy gelatin. Often occur in long threads and form rhizoid colonies. Form of rod usually not greatly changed at sporulation. The type species is *Bacillus subtilis* Cohn:" and genus 2: "*Clostridium* Prazmowski 1880 Anaerobes or micro-aerophiles. Often parasitic. Rods frequently enlarged at sporulation, producing clostridium or plectridium forms. The type species is *Clostridium butyricum* Prazmowski."

Breed, Conn and Baker (1908) commented at length on the major divisions proposed by the Committee: "This family, for the spore forming rods, has very good justification. The two genera, *Bacillus* and *Clostridium* can probably be separated, but whether on the basis of relation to oxygen or of shape of the sporangium, the future must decide. Although relation to oxygen is a very important physiological distinction, it must be admitted that the selection of a physiological basis for the separation of these two genera is rather unsatisfactory. It places some of the polar-spored organisms in one genus, some in another, and raises the question where to place facultative anaerobes like *B. mycoides* and *B. cereus*." These authors proposed a family (5): "*Bacillaceae*, rods producing endospores, usually Gram-positive. Flagella, when present, peritrichous. Primarily saprophytes secreting proteolytic enzymes. A few parasites." This family would include the majority of the anaerobes and many aerobes.

Buchanan (1918, a and b) reclassified the bacteria, using physiological characters far less than did the Committee. His classification of the non-acid-fast members of the family *Bacteriaceae*, sporogenous rods, is as follows: Tribe 1. *Bacilleae*, endosporogenous rods, with four genera: 1. *Bacillus*, Aerobic rods, usually Gram-positive, as a rule liquefying gelatin, spores usually not distorting rods when formed. 2 and 3. Anaerobic or micro-aerophilic usually: 2. *Plectridium*, spores produced at

extreme tip of cells, forming typical drumsticks. 3. *Clostridium*, spores not produced at extreme tip of cells, at least not forming drumsticks. Cells usually somewhat swollen when spores are formed. 4. *Metabacterium*, with usually a number of spores within a swollen cell. Tribe 2. *Bacteriaceae*, not producing endospores.

Rahn (1920) defines the anaerobes as sporulating rods that store up Iogen (granulose) with or without glycogen. He believes that further research would show the possibility of changing any spore-forming anaerobe into another.

THE CHARACTERS USED FOR CLASSIFICATION

Let us consider the value in classification of the characters whose use has been proposed in the above arrangements.

Morphology of the vegetative cell. Most of the authors define the *Bacillaceae* as rods. Exceptions to this rule are the arrangements of Zopf (1885) who believed in the transformation of bacterial species, and of Jensen (1909), who used physiological characters for his classification. Both Zopf and Jensen state that spherical forms may be included in such a family. Apparently the unity of origin of the cocci has never been settled by systematists. Winslow and Winslow say (1908): "Yet a consideration of the properties of the members of the group makes it clear that they are mutually interrelated and all sharply separated from the rod-shaped bacteria, except perhaps at one end of the series which they form." Breed, Conn, and Baker do not consider the question of the unity of the origin of all spherical bacteria as settled. The series of strains which Winslow and Winslow used for their study did not, I believe, include any strict anaerobes. Many cocci are facultatively anaerobic. Strict anaerobes of this group are only occasionally met with, but it does not follow that their occurrence is very rare, because the usual technique for the isolation of anaerobes involves some heating process that eliminates the non-sporulating organisms. Anaerobic cocci have been described by a number of authors. Ozaki reviewed the subject (1915). There are, in his list, four

diplococci, three micrococci, and four staphylococci, one of these latter appearing at times as a streptococcus or as a micrococcus. Anaerobic streptococci are, according to Hüssy and Bondy, normally human saprophytes which may become parasitic. They are commonly found in cases of puerperal endometritis, according to these authors and according to Schottmüller. Adamson isolated anaerobic diplococci from eighteen out of fifty-one wounds. Sternberg has described an anaerobic streptococcus. Anaerobic cocci have been found in the blood of scarlet-fever patients by Dick and Henry and in that of measles patients by Tunnicliff. Beijerinck (1906) finds a sarcina in soil that is a fermenting anaerobe. Winslow and Winslow did not find the arrangement of the cells of the cocci a good basis for classification. About half the anaerobic cocci produce gas in sugar media. Probably nothing can be decided as to the real affinities of these anaerobic cocci until the chemical behavior of some of them has been studied by one worker and has been compared with that of the anaerobic rods. A morphological basis, when one considers the varied types of anaerobic cocci, would certainly lead one to conclude that anaerobiosis had been independently acquired by certain strains of the various types. Here, as in many cases, morphological and chemical criteria flatly contradict each other. But form of cell (sphere, rod, or spiral) is certainly to be recognized as a much more fundamental character than is the arrangement of cells. Nevertheless, Almquist found that bacilli may grow as spheres at low temperatures. Yet it would seem that the anaerobic cocci do resemble other cocci in their chemical behavior more than they do the anaerobic rods (see Adamson, 1918–19, p. 394). So for the present it is advisable to exclude them from an anaerobic group which contains rods. The chemical study of the anaerobic cocci should be more extensive before they can be placed anywhere.

Motility. The possession of flagella was used as a primary character for classification by Migula, spore-formation being given a secondary place. Chester followed Migula in crediting importance to this character; but other workers have not done so. The Committee (1920, p. 516) state: "The prominent place

given to motility seems to us to constitute a peculiar infelicity in these schemes." And Winslow and Winslow (1908, p. 52) do not find motility correlated with other characters in the group of the *Coccaceae*. It would seem that for our purposes the character of motility was entirely unsuited for the making of major divisions. Thus organisms in general so similar as *B. Welchii* and *B. Chauvoei* were placed by Migula's system in entirely different groups because one was flagellate and the other was not. And aerobes of many sorts and proteolytic anaerobes dwell in his work peacefully side by side with *B. Chauvoei* in the enormous and unwieldy genus *Bacillus;* while *B. Welchii* on account of its nudity is relegated to the genus *Bacterium* along with *Bacterium tuberculosis* and other strangers.

Spore formation has been turned to by many classifiers as an important character for the subdivision of the rod-like forms. Zopf, Kruse, Fischer, Lehmann and Neumann, the Committee, and Breed, Conn, and Baker have used it as a basis for making their primary division. It is probable that this is a character of far more value than is motility. It is, however, true that, though there are many similarities between the sporulating rods, we have no proof that they are more closely related to each other than they are to some of the non-sporulating rods, or that the formation of spores originated with any one type. Should we accept such a hypothesis, we should still be unable to show that certain sporulating rods had not lost their power of spore-formation. This power is certainly an advantage to a species, and on that account anaerobic forms losing it are not so likely to persist as are others. But that does not mean that such a phenomenon may not occur.

Kruse strenuously protests (1896, p. 81) the use of spore-formation as a primary character. Moreover the adoption of spore-formation as a character for the subdivision of the rods would make us exclude from the anaerobic group such organisms as *B. egens*, *B. necrophorus*, Bacillus D of Adamson, *B. fragilis* of Veillon and Zuber, and probably a goodly number of undescribed organisms which in their behavior closely resemble the sporulating anaerobes. I find that *B. egens* and another Gram-

positive pathogen are so similar in many respects to *B. Welchii* that they might easily be identified as one species by many workers, and it is evident that only an artificial classification would separate them, yet *B. egens* and the other pathogens have not been shown to produce spores. *Asporogenous* anaerobic rods have not been frequently reported, but when one remembers that the preliminary step in isolating anaerobes is usually a heating process, it will be clear that the proportion of anaerobes that do not sporulate may be considerably greater than one would estimate on the basis of published descriptions. The soil mixtures from which Weinberg's organisms, *B. egens* of Stoddard, and my above-mentioned pathogen were isolated had all been subjected to a physiological weeding out in human tissue before they were inoculated into media, and a colony method without heating was thus practicable for isolation purposes.

The matter of a primary division of the rod-shaped bacteria then simmers down to a question of whether an anaerobic habit or a spore-forming habit is the more fundamental one. It is perfectly evident that certain asporogenous anaerobes have closer physiological affinities with certain sporulating anaerobes than the latter have with the sporulating aerobes or even with most other anaerobes. We may have in the power of sporulation such a phenomenon as that noted among the insects: there are primitively wingless insects, the *Thysanura*, and there are various types of insects, such as the *Siphonaptera*, and the *Mallophaga*, which have lost their wings, and there are insects that have no wings at the time or in the form that we happen to observe them—larvae and pupae and worker ants and apterous mutants of winged forms. Therefore the possession of wings, conspicuous insect characteristic that it is, has been discarded as a character for the separation of insects from other forms. The whole question is reduced to the much agitated one: Are we going to give precedence to physiological or to morphological characters in the classification of the bacteria?

Morphology of the sporangium has been used as a character by a long succession of workers. Its use may be more vigorously attacked than that of the other characters. It was introduced

by strict morphologists (Zopf, Fischer) entirely independently of any physiological criteria, and in this way was quite justifiable because by its use a superficially consistent division could be made. The rods which did not swell at sporulation formed one group, those which did swell formed another. But Kruse and the Committee have superimposed upon this type of classification a physiological one, and the result is a division that it is impossible to carry out. Probably more aerobic rods fail to swell at sporulation than do anaerobic rods, but the exceptions to this rule are so numerous on both sides as to render worse than useless the employment of the morphological character in connection with the physiological one. The reader is referred to the illustrations given by Ford and his co-workers and by von Hibler (1908) and by the Medical Research Committee.

The position of the spore has been used by several authors in subdividing the anaerobes. Species of anaerobes have very characteristic ways of sporulating. But the position of the spore may vary in one species within limits wide enough to render its use exceedingly inadvisable as a character for the grouping of genera. One may take as a single illustration the behavior of a pure strain of vibrion septique. Vegetative forms are fairly uniform on most media. Sporangia, however, show in their variations all the characteristic forms described by Fischer, by the Committee, and by Buchanan. They are, on meat medium (forty hours' culture), usually thickened in the center with the typical form of clostridia. But some rods may contain spores and still have parallel sides, and forms with sub-terminal and terminal spores are nearly always to be found. On serum media the vegetative rods may vary greatly in their proportions, the sporangia assume many fantastic shapes, and "drumstick" forms are common. On the liver of animals the vegetative rods form enormous thick filaments: some strains may sporulate without at all changing their outline or may form clostridia, and some, identical with the first in morphology on ordinary media, may, on the liver of animals, show club-shapes that resemble the clubs formed by the actinomycetes, while others form great globoid masses, terminally or mesially placed in the rods. Rarely

three or four spores may occur in a rod which has remained undivided. In fact the only fixed morphological character to be noted is the shape of the spores, which, so far as I know, in this species everywhere remains oval. This is the sole morphological character that was noted in my anaerobic studies that cannot be assailed as inconstant, yet the shape of the spore is a character that has been consistently overlooked by classifying morphologists, who have chosen instead the extremely variable one of spore-position.

The size of the rod has not been mentioned by most classifiers. In general the sporulating rods are larger than the non-sporulating rods. But to reduce this generalization to definite measurements as Lehmann and Neumann have done is not a practical procedure.

The arrangement of the bacilli in chains is not significant. Probably the aerobes form chains more frequently than do the anaerobes, but filament formation cannot logically be used as a character for their differentiation. Winslow and Winslow found chain formation a character of minor value in the classification of the streptococci. Certain organisms, e.g., *B. Novyi*, regularly form filaments on certain media, but the character is of specific, not of generic, value.

Morphology of colonies would be mercilessly discarded by experimental workers as a means of subdividing a large group like that of the *Bacillaceae*. It has more value for lower subdivisions.

The Gram-stain is an impossible character to use in dividing the *Bacillaceae*. It fits neither with the morphological characters nor with the physiological. There are numerous Gram-positive anaerobes and aerobes, and numerous Gram-negative anaerobes and aerobes. (See Heller, 1920.)

Granulose (Iogen) content of the bacterial cells cannot be seriously considered as a general character of anaerobic rods.

Habitat. Winslow and Winslow found that the *Coccaceae* could logically be divided according to habitat. The parasitic forms constituted one group and the saprophytic ones another. Certain anaerobes are frequently inhabitants of the intestines

of animals. But this type of character has not been worked out for the anaerobic organisms and should evidently not be used in classification until it has been investigated thoroughly. In view of the fact that a large variety of anaerobes are to be found in soil it is not advisable to state that anaerobes are "often parasitic." Habitat might be used conservatively as a descriptive character.

The formation of toxin and the pathogenicity for animals—the most interesting of characters to the majority of us—cannot logically find a place in the higher divisions of our group. They become of more systematic value in classifying genera and species, but they should always be used in connection with other characters. Jensen's grouping of *B. tetani* and *B. botulinus* in the genus *Botulobacillus* because both produce toxin is not advisable. The toxins produced by these organisms are diametrically opposite in their effect on nerve tissue and that of *B. botulinus* and probably that of *B. tetani* are entirely adventitious so far as a parasitic mode of life is concerned. Other anaerobes of different affinities form toxins that produce still other and different effects.

We have seen with what ease objections may be made to almost any morphological character used for the division of the rods of higher metabolism, in case any physiological character is allowed to enter into the classification. We have stated also that some of the proposed morphological characters are not sound for single species or even for a given culture of a single strain. As Breed, Conn, and Baker say, the future must decide what type of character, physiological or morphological, will predominate in the classification of the bacteria. The two systems are so often contradictory that they can never exist side by side. One must always be used as the chief deciding factor, the other as an auxiliary which may, at any time, give precedence to the former.

Experience with a single group may be misleading. The higher plants, and even the fungi, may be satisfactorily classified on a purely morphological basis. At present the systematics of bacteriology are so tentative that the matter must be left to

the judgment, or shall we say to the taste, of those who have themselves worked with the groups that they discuss. I am totally unable to see how morphological criteria can possibly be used to any logical end in the classification of the rods of higher metabolism, or even of the main groups of anaerobes. In 1902 Achalme found morphology of absolutely no use for the differentiation of anaerobes. In 1905 von Hibler energetically decried the use of morphology in anaerobic classification. In analysis of the anaerobic group morphology has its place, and can logically be used to distinguish types that are otherwise similar. It can *not*, in my opinion, be used to unite groups that are otherwise dissimilar. In the anaerobic group morphological criteria alone would hopelessly bewilder the student and lead him to the correlation of fundamentally different types and to the separation of sister rods of the same strain. Morphology need, however, never be entirely discarded from classification. The morphology of the anaerobes is, for a given species, so characteristic, that if it be observed conscientiously, and if the worker does not generalize too freely in formulating his description, it may well be used as a valuable descriptive character for species, and as an auxiliary character for the description of genera. It is in organizing the major groups of anaerobes that morphology fails us. Professor Harvey M. Hall of the Botany Department of this university suggests that after a logical and fundamentally historical chemical classification has been made, morphological characteristics will be found which will be consistent with it. One must distinguish between different types of morphological criteria. Gross form of rod and position of spore are not fundamental morphological characters: they vary greatly within the species. But a highly refined cytological technique such as has never been generally applied to our organisms might reveal consistent morphological characters.

THE ANAEROBIC RODS

In my opinion the most logical division of the bacterial rods—the rods which split higher compounds and are not acid-fast—is the physiological one of susceptibility to free oxygen. Ability

to live in the absence of free oxygen has been developed by too many types to make it a character of value. But fewer types have developed a susceptibility to free oxygen. The classification proposed by the Committee (1920) places certain anaerobic forms such as the anaerobic leptotrichia with aerobic forms that are patently their relatives. The Committee justly assigns generic rank to the obligately parasitic, non-sporulating, shyly growing, fusiform anaerobes. They do not mention the anaerobic cocci, which should probably be included with the *Coccaceae*. The Committee does not mention any anaerobic non-sporulating, non-fusiform rods. In my collection there are two such strains (*B. egens* and one of my own isolation) which do not readily attack milk, and these organisms do not fall into any of the groups designated in the key, which follows the Committee's classification. *B. necrophorus*, again, of whose phylogenetic position I am in doubt, does not sporulate.

Fusiform bacilli have probably recently acquired an anaerobic habit through parasitism. Thus Larson and Barron describe a strain of these organisms which became adapted to growth under aerobic conditions. Analogous is the behavior of *B. abortus-bovis*, which frequently refuses to grow aerobically when first isolated but later accustoms itself to living in the presence of oxygen. The anaerobic habit may, in some cases, be due to a sensitiveness to carbon dioxide instead of oxygen. Curtis has described a motile curved anaerobic rod which he isolated from uterine discharges. The phylogenetic position of this organism is in doubt. So also is the position of the branching anaerobes *B. ramosus* and *B. furcosus* of Veillon and Zuber, and the influenza-bacillus-like rod isolated from an abscess by Russ. Tunnicliff reports anaerobic rods from rhinitis and from bronchitis patients; and Tunnicliff, Plotz and his co-workers, and Dick and Henry, report anaerobic organisms in the blood of fever patients. But these organisms grow slowly and do not resemble the chemically active anaerobic rods. We are justified in concluding that an organism which has lived as a saprophyte or parasite in the tissues or in the uterus may owe its anaerobic habit to such residence. There are several reasons why we should

hesitate to attribute to a parasitic or intestinal saprophytic history the anaerobic habit of the rods found commonly in soil. These rods are abundant in unmanured soil, their species are very numerous, their metabolic processes exceedingly varied. They may grow under aerobic conditions in company with aerobes and may grow in the presence of oxygen in pasty or solid material or in liquids containing soap or other substances which alter surface tension. But they retain their anaerobic habit on clear liquid or agar media. The commonest intestinal organisms, those of the colon group, have not assumed a sensitiveness to oxygen. Many of the anaerobes, such as those of putrificus, sporogenes, and bifermentans affinities, are the common agents of putrefaction outside the animal body, while others described by Omeliansky are the common cause of the decay of cellulose. When parasitic outside the intestine, these organisms usually show little of the character of true parasites, but cause fulminating fermentative processes which do not pass from affected individuals to healthy ones. *B. abortus*, the anaerobic streptococci, fusiform bacilli, and certain types of *B. coli*, when they invade the tissue may establish chronic infections, characteristic of highly developed parasites, but the anaerobic rods common in soil do not, so far as we know, behave in this manner. They are apparently unable to establish themselves as chronic parasites in tissues which are well vascularized. Had they a history of intestinal saprophytism, we should probably find highly adapted parasites among them, and should find it easy to educate them to an aerobic habit.

No one has, of course, suggested that the nitrogen-fixing anaerobes described by Winogradsky developed their anaerobic habit through parasitism. These organisms are active splitters of carbohydrates. They are usually regarded as primitive. It is more probable that the anaerobes of higher metabolism had an evolution of the following type rather than one from the sporulating aerobes or from intestinal saprophytes of large animals which appeared at a comparatively late geological period.

Nitrogen-fixing anaerobes that split carbohydrates. E.g. *Clostridium Pastorianum* Winogradsky, a large anaerobic rod that forms oval spores.

Carbohydrate-splitting anaerobes that can utilize fixed nitrogen but not free nitrogen. They do not produce gross proteolysis. E.g. *vibrion septique.*

Anaerobes that split proteins very actively. Some but not all have lost the power of splitting carbohydrates. E.g. the sporogenes type.

Geologically this sequence would be the most natural. But we know so little about bacteria and their evolution that any evolutionary arrangement is little more than guess-work at the present time.

It is my intention to propose a division which seems more logical than "the *Bacillaceae*, spore-bearing rods," as distinguished from the "*Bacteriaceae*, non-sporulating rods of higher metabolism." This division implies the creation of a family: "The *Clostridiaceae*, rod-like forms, not spiral, which will not grow within seven millimeters of the surface of a shaft of clear tissue-free agar medium contained in a tube 12 millimeters or more in diameter, incubated in air, in which they are able to grow in the depths. They may or may not possess peritrichial flagella; they may or may not form spores. Most members of the group are characterized by their energetic action on proteins or on carbohydrates or on both of these types of substances." It would be unwise to claim that we have evidence to show that these organisms are descended from a single type—in other words that this is a perfectly logical classification. Bacteriologists have no characters available for purposes of classification whose nature is sufficiently understood to grant us the liberty to make such assumptions. But I believe that this primary division will separate fewer types that are physiologically alike than any other thus far proposed. The energetic action of the anaerobic non-fusiform rods upon carbohydrates and proteins is characteristic and separates them from most other groups. In the present state of our knowledge it is only the separation of types that have several characters in common that is care-

fully to be avoided. The bacterial characters understood by us are so elementary that we can, as yet, have no assurance that we are not at times uniting types that have not the same ancestry.

It will be noted that the Committee has arranged the bacteria into orders, families, and genera. Most families have also been divided into tribes. The genera of the *Actinomycetales* have not thus been arranged in tribes, because their relationships are avowedly obscure. But the sporulating rods have been given the very inferior position of two genera and the tribal relationships are not expressed. This is because these organisms have been so slightly studied. As Ford says in his introduction, our knowledge of the spore-bearing bacteria is still in a state of chaos. The sporulating organisms, at least the anaerobes, are legion in species, and form a group that is to be divided and subdivided.

Whether or not the aerobic spore-bearers (genus *Bacillus* of the Committee) form a homologous family, I am unable to say. Compared with the anaerobic rods they are apparently very few in number of species. Ford and his co-workers list twenty-eight species which they place, on the basis of morphological and gross cultural characters in nine "groups." These groups would probably form as logical genera as some of the others which have been recognized.

It may be asked why tribal rank should not be assigned to the anaerobic and aerobic rods instead of family rank. It would seem that the group of anaerobic rods is sufficiently large, peculiar, and important, to warrant its being given family rank. Probably none of the botanical or zoological families contain nearly as many species as may be found among the anaerobic rods. On strict analogy with botanical and zoological classifications the anaerobes should command an order at least, but being unfortunately dogmatically confined in our classification to a single class which must include all one-celled cellulose- and chlorophyll-free plants that divide by simple fission, we must be modest in our demands.

In order to ensure a natural classification, characters must be worked out for each group, characters that will to some extent correspond, and show by such correspondence or by the lack of it where lie the historical divisions and where the parallel developments that have taken place independently. This alone is a great labor. For the group of the anaerobic rods and for many other groups nothing of the sort has as yet been seriously attempted. The Winslows' classification of the *Coccaceae*, a pioneer work in this direction, has applied several principles, which may well be heeded in making future classifications. These authors applied to 500 strains of cocci from various sources the biometric principles in use by students of heredity, by anthropologists, and to some extent by botanists and zoologists. Upon a study of the tabulated figures based on the behavior of these organisms they formulated their determination of what to call a species and of how to group species into genera. They found, in common with botanists and zoologists, that when abundant material is at hand it is quite impossible to define as a species one single type. If our methods were sufficiently refined we could probably distinguish every bacterial strain from every other, just as we can distinguish every human being from every other. A species is finally to be determined by comparing the characters of aggregates of individuals (higher plants and animals) or of strains (bacteria), and by selecting the types which occur most frequently as the standard upon which to base specific descriptions. The conclusions arrived at by Winslow and Winslow as to analysis of their data are as follows:

> First, each center of numerical frequency, marking a group of organisms varying about a distinct type in regard to a single definite property, may be recognised as a species. Second, those species which are bound together by the possession of a number of similar properties may be constituted as genera, and larger groups of genera, still characterized by some characters in common, may receive the rank of families or subfamilies.

This method of working is evidently very different from the old method whereby one man described one strain and another

man another, and a third decided some ten years later from their descriptions whether they were working with the same or with different species. The biometric method is evidently true biology, while the other is a process of cataloging. The principles of the biometric method are those that one would choose to follow, even though one were unable to make a study of so extensive a series as did Winslow and Winslow. But it is upon the first method, that of collation of descriptions from the literature, that our comprehensive classifications have so far been made. This has led to a complete misunderstanding of the nature of the anaerobic group. A *few* anaerobes have been described, most of the descriptions being wholly inadequate for purposes of specific determination. This fact has in no way deterred workers from making identifications. Some of these mistaken identifications are now thoroughly ingrained in the literature, for example the use of the name *putrificus* in Germany for the *sporogenes* type of organism, when there is a different definite type existent which corresponds far more closely to Bienstock's description of *B. putrificus*. The names of some of the described anaerobes have been accepted, and if these types are pathogenic or very common they find their way into the textbooks. Textbooks mention usually five anaerobic organisms: *B. tetani*, *B. botulinus*, *B. oedematis-maligni*, *B. Welchii*, and *B. Chauvoei*. So far as I can see the classifications are largely based on a conception of the anaerobic world which knows few forms but these. But the worker with "wild" material can easily pick up and isolate two or three new species of anaerobes a day for an almost indefinite period. Few workers now pay any attention to non-pathogenic anaerobes, knowing that their path would be crossed by so many new species that no end but the mere description of new species would be attained. But these undescribed forms are just as important, theoretically, to the systematist, as are the pathogenic ones.

The ideal way of classifying anaerobes would be a biometric one carried out on a scientifically adequate number of strains. But it will be years before sufficient interest in the anaerobes exists to warrant the collection of any such material. The labor

of making such collections and of keeping close watch on all strains to insure their purity is tremendous. The bacteriologist is not the only systematist who has to do with such a problem as ours. The classifier of the *Coccaceae* is in the position of the curator of a museum who has before him the skins of a hundred or two of squirrels or other rodents, their measurements and habitat given, their skulls freed of muscle reposing in tiny bottles by their sides. The classifier of the anaerobes is today in the position of the exploring zoologist who sets his traps at night on his journey and catches one or two or three new rats or mice that do not resemble any thus far met by him. Both men describe new species and both serve science in so doing. But the museum worker may use as his type-species the animal whose characters are an average of those of all the rest, while the exploring zoologist must call the "type" one of his chance catches which may be a freak in one or more ways. And yet we would not have the explorer place his mice from a far country nameless in a museum for a future zoologist to describe some seventy-five years hence when the far country has been settled and the mice have been caught by the hundred.

The problems presented in the classification of the *Coccaceae* and of the *Clostridiaceae* are quite different in other ways. The anaerobes form a group of far more diverse types of organisms, both from the morphological and from the physiological standpoint, than do the cocci. One may say that their characters are more salient, more easily perceived, or more definite in their nature, than are those of the cocci. Or one may state with equal truth that the anaerobic group is a less homogeneous one than that of the cocci. One would also be justified in stating that the anaerobic species and genera are far more numerous than are those of the *Coccaceae*. Therefore a representative and adequate collection of anaerobic strains for statistical study would have to contain not hundreds but thousands of strains. But this element of distinctive characters places in our hands a means for the determination of genera before we are familiar with many strains of each genus.

SPECIFIC AND GENERIC CHARACTERS

No one will dispute that the decision as to the line between specific characters and generic characters lies with the systematist who, though he have a previous knowledge of many other groups, has confined his attention to one group, and not with the systematist who organizes the published work of others. In different groups these boundaries vary somewhat. But in general the following definition will probably be accepted for such types of material as we are *unable* to examine thoroughly by a biometric method. *Strains of bacteria that regularly and consistently differ from each other in certain characters that we have come to recognize as significant may be assigned to different species. These characters may be quantitative in their nature. It lies in the hands of each worker to decide what the value of these characters is.* An arrangement made without reference to biometric data is in any case bound to be tentative. *Generic characters are based on qualitative properties.* As a working system the following classification of specific and generic characters for organisms of the anaerobic group is suggested:

Generic characters, qualitative:
- Qualitative chemical action: behavior on usual laboratory media (excepting the fermentation of milk).
- Staining reaction and general morphology of individuals.
- General habits of colony formation.
- Pathogenic action.

Specific characters, quantitative:
- Quantitative chemical action: behavior on carefully standardized media; hydrogen-ion end point attained as a result of specific enzyme action.
- Sugar fermentations if not subject to active mutation.
- Peculiar habits of the morphology of individuals.
- Exact behavior of colony formation on a standard medium.
- Details as to pathogenic action.

In studying my material, I find abundant justification for the application of properties of this sort. With such characters to build upon, a more detailed structure, more exactly expressing

relationships, can later be erected. We are not yet ready to declare where the limits of variation for the organisms of our group may lie. But I am decidedly of the opinion that in general the old conception of species as accepted for the anaerobic group must in future be taken as the conception of genera, and that we must be more exact in our examination and analysis of these organisms. To do otherwise, and classify as the same species organisms which regularly and consistently show marked quantitative differences in their behavior, would be to distinguish our system of classification sharply from those of the botanists and zoologists and to set up our own meanings of "species" and "genus." The application and use of the characters here suggested will be described more fully in a future paper.

Perhaps the first-noted definite "character," splitting up a so-called species into a number of groups, is the agglutination reaction. Thus Tulloch by this method demonstrated the existence of four types of the group recognized under the name of *B. tetani*. Robertson thus subdivided her vibrion septique strains into four groups, and Henry divided the species of *B. sporogenes* into two groups on the basis of the agglutination reaction. It has been found by various workers that agglutinating anti-sera formed against various strains of *B. Welchii* do not agglutinate heterologous strains of the same organism, though Werner found a serum that agglutinated one out of several heterologous strains. When one thinks, however, of the comparatively numerous cases of cross agglutination recognized in other groups, some of which may, and some of which may not be modified in their importance by absorption-of-agglutinin tests, and when one considers the Weil-Felix reaction, one is ready to look for a more highly specific character than that of the agglutination reaction by which to analyze his strains. The Medical Research Committee term the agglutination reaction "ultra specific" (1919). I personally regard this reaction as of sub-generic rank, and not as of sub-specific rank in the anaerobic group. The agglutination reaction has not yet been investigated thoroughly enough to determine its value as a systematic character for anaerobic bacteria.

NOMENCLATURE OF LOWER GROUPS

Our next concern relates to the nomenclature of our genera and species. We are peculiarly hampered in bacteriological work, when we try to base our names for organisms upon their behavior or characters. Morphology is a notoriously bad bacterial character for generic names, though it has long been used as a generic character for our primary divisions of the bacteria (*Coccus, Bacillus, Spirillum*) and apparently has a sound basis in this case. Pathogenic action is an equally misleading character upon which to base generic names: most anaerobes are not pathogenic and of those that are, various groups produce gas, oedema, haemorrhagia, etc. Chemical action would be the best type of character for descriptive purposes. But how often might we not, in a group that is so enormous as that of the bacteria, inappropriately name a new genus for a chemical character that was possessed in a greater degree by other genera, or was not possessed by all the members of the genus? There is also a prejudice among botanists against the formation of generic names from specific names, though such forms are not unusual in zoological nomenclature.

Dr. Karl F. Meyer has suggested to me the use, for purposes of generic nomenclature, of patronymics, preferably of the name of the author first describing the original species of a genus. This seems to me the most fitting and logical procedure. It has ample precedent in botanical nomenclature, and has been used in bacteriological nomenclature for years: e.g., *Pasteurella*, *Eberthella*.

Recommendation V.e. (International Rules for Botanical Nomenclature, Chap. III, Sec. 3, No. 3) will, if heeded in the formation of generic names, aid greatly in overcoming conservative objections to the new system of classification. This recommendation reads: "To recall, if possible, by the formation or ending of the name, the affinities or the analogies of the genus." Thus in the group of the cocci, *-coccus* has been accepted as the usual termination of the generic appellations; *-bacillus* has never been popular for such formations, probably on account of its

length. I had considered the ending *-ella* as used in 1900 by Lignières for *Pasteurella*, and by Buchanan for *Pfeifferella*. But *-ella*, like *-ia*, is a common ending for generic names among the higher plants. Would it not, in view of the existence of this recommendation, be appropriate to terminate the generic names of bacillary forms with the ending *-illus* (from *Bac-illus*)? (Patronymics ending in vowels may drop the final vowel before adding *-illus*.) Perhaps the ending *-erium* may also be found appropriate for names created in subdividing the old genus *Bacterium*. Specific names should be adapted from the original specific name, if such is valid, and new species can, of course, be named according to the will of the author describing them.

TYPE STRAINS

Because of the factor of variation in the habits of cultures, because many species of organisms must be frequently transplanted to keep them alive, and because of the important rôle played by contaminations, the custom, so long accepted by the botanists and zoologists, of preserving in museums type-specimens of newly-described species, has never been popular with bacteriologists. The facts as related to the anaerobic group are as follows: No experienced investigator of anaerobes would care unreservedly to turn over to anyone else his type-strains for general distribution, because of the ease with which they may become contaminated, and because of the difficulty that the ordinary worker has in recognizing contaminations. The rather generally disseminated view of Grassberger and Schattenfroh (see Heller, (1920)) that the characters of anaerobes are highly variable is one to which I cannot subscribe. This view, which has cast a blight on modern German anaerobic studies and caused grave misinterpretations (see Rahn), has also deterred workers from the use of type-strains to make their descriptions definite. The anaerobic bacteria are fairly stable types (when in pure culture) and they have, in common with all other organisms, that degree of variability which permits them to adapt themselves somewhat to changing conditions and they

may, occasionally, show mutations as do all living organisms. The problem of their variability is essentially no different from the problem of the variability of other bacteria. Their behavior toward proteins is remarkably constant, while their action on carbohydrates is somewhat variable.

THE SUBDIVISION OF THE CLOSTRIDIACEAE

We have now outlined the status of the anaerobes in bacterial classification, and the position to be held by genera and species. It remains to organize the structure between the generic rank and the family rank. It is here that we have the most need of allowing room or elasticity for the convenience of future systematists whose information will be greater than ours is today. With our present knowledge I do not think that we are entitled to make more than one main subdivision of the *Clostridiaceae*. This division should follow that made by von Hibler in 1899, in 1905, and in 1908. Von Hibler showed that some anaerobes produce more acid than alkali on certain media, while others produce more alkali than acid. On the basis of this observation he classified the fifteen species studied by himself into two groups. He titrated brain cultures and milk cultures against $\frac{N}{100}$ HCl and KOH, and found that on both media the organisms of the first group produced an acid reaction, while on brain medium, which is poor in sugar, the organisms of the second group invariably produced an alkaline reaction, and on milk, though some of them at first produced an acid reaction, they all finally gave an alkaline end point. The production of an alkaline reaction was always associated with peptonization of milk and was usually associated with a blackening of brain medium and with the production of hydrogen sulfide. The organisms that produced and maintained an acid reaction in milk and brain never peptonized casein or blackened the brain particles.

The division thus made by von Hibler has been accepted and followed by Jensen and by various anaerobic workers. The alkali-producing group is termed *proteolytic* or *putrefactive*, the

acid-producing group *non-proteolytic* or *saccharolytic*. The organisms studied by von Hibler were all energetic in their reactions. *B. Novyi* and organisms related to it, such as *B. oedematiens*, form hydrogen sulfide in blood media and do not produce much acid in milk. They do not peptonize casein or blacken or putrefy meat. They and the *Bifermentans* type and certain other organisms that I have encountered do not fit so nicely into von Hibler's scheme that we can safely place them in either group without drawing a dogmatic line and measuring their activities accurately. Douglas, Fleming, and Colebrook describe a sporulating anaerobe, *B. cochlearius*, which shows no marked properties that unite it with either of von Hibler's groups. It therefore seems advisable to define conditions under which anaerobes may be tested to determine their affinities with these two groups. Objection may be made to such a separation of the anaerobes on the grounds that when borderline organisms are in question it is but a cataloging process to separate them. Perhaps so, but when a classification is developing as is this one and when so very few borderline forms are known, a cataloging classification is better than none; later a group containing these organisms may be formed if necessary. Moreover the vast majority of anaerobes do fall definitely into one or the other of these two groups and can be placed where they belong on the basis of their behavior on ordinary media.

The requisites governing the selection of a medium for such a purpose are definite. In the first place all anaerobes must grow upon it. Blackleg organisms usually refuse to grow on simple sugar media. In order to give the organisms a good start, and to furnish material for the formation of acid, our medium should contain a little sugar, of the sort available to the greatest possible number of anaerobes: glucose is probably the sugar which best fulfills this requirement. But this sugar should be little in amount, and sufficient protein should be present, so that the acid formed from the sugar may not inhibit the growth of the organisms and prevent them from carrying the reaction back to an alkaline end point if they are capable of so splitting protein that they produce such an end point in the absence of acid.

Probably tissue-containing media best fulfill the above requirements. They need no more glucose than that which they derive from the tissue contained in them. Brain medium as used by von Hibler would be excellent were it made up with sufficient liquid for titration purposes. Von Hibler titrated liquid from his brain medium with litmus against potassium hydrate or hydrochloric acid. Today workers would prefer to use a hydrogen-ion determination, for which a fairly clear liquid is necessary in case the simple colorimetric method is employed. Bromthymol-blue is a suitable indicator for making this separation. At present most laboratories use the beef-heart medium introduced by Robertson and Martin for anaerobic study because it has many technical advantages over brain medium. It should preferably contain about 5 per cent of peptic digest broth and should be made with twice its weight of distilled water and tubed in large tubes in order that enough liquid may be present for hydrogen-ion determinations on several occasions.

Ten days' incubation at 37° will be found quite sufficient in most cases for such a determination as we wish to make. Von Hibler found the reaction decidedly acid or alkaline in brain medium after five days. But to allow time for weakly proteolytic organisms which form acid from glucose, to carry the reaction over to the alkaline side, we should incubate the cultures for twenty days at least. Such organisms do not at first produce gross signs of putrefaction, but their proteolytic tendencies may be tested for by the lead acetate test for hydrogen sulfide. There may be soil anaerobes which do not grow at 37°. Time and temperature for incubation will have to be decided upon for such organisms separately in case they are found.

Von Hibler (1908, p. 88) found that with his pasty brain medium the mode of incubation, aerobic, or anaerobic in hydrogen or in carbon dioxide, made no difference in the reaction. But if we are to use a medium with a considerable amount of liquid on the surface it would probably be unwise, even though anaerobes grow in the medium with the surface open to the air, thus to incubate our organisms for twenty days. An anaerobic method should be employed. The simplest method available

to most laboratories is stratification with vaseline. After incubation the culture should always be boiled to expel carbon dioxide.

For pure culture study the exact point of the reaction of the medium at the time of inoculation is not important. It is important only that the reaction should be well within the limits for growth of the organism studied, and not very far from neutrality. A reaction of pH 7.2 (neutral to litmus) has been used in this laboratory for most anaerobe media. The selection of a reaction point for the dividing of the two types of anaerobes is more difficult. Von Hibler (1908, pp. 89, 104) used the neutral point of litmus. The selection of a dividing point in meat medium is frankly an arbitrary affair. I suggest pH 7.0, the neutral point of hydrogen-ion concentration, for the dividing line between the alkali-producing and the acid-producing anaerobes.

It is unlikely that any other division of the *Clostridiaceae* will be made which would be placed above the division into the two groups just described. But I am certain that various arrangements of the genera which compose these groups will in time be made. There are many anaerobes that behave quite alike in one way and entirely differently in other ways. If this were to be a final classification of the group, or if one wished to outline a temporary classification, one would give the division into putrefactive and non-proteolytic anaerobes a tribal rank. But I am quite certain that the makers of future arrangements will want very much to utilize tribal and subtribal ranks for other purposes. I had myself thought of dividing the non-proteolytic anaerobes into two tribes or subtribes on the basis of presence or absence of flagella, but Dr. M. Christiansen has repeatedly been unable to demonstrate flagella on his whale-septicaemia bacillus which so closely resembles the vibrion-septique type of organism that they should probably be placed in the same genus. But tribal and sub-tribal classifications are sure to be made by someone before long and it will be a better provision for the future to give the proteolytic-non-proteolytic division sub-family rank.

I propose, therefore, the two subfamilies, *Clostridioideae* and *Putrificoideae*.

Clostridioideae: Clostridiaceae which on meat medium produce after twenty days' incubation under oil at 37° a reaction of pH 7.0 or a more acid reaction, the reaction being read after the culture has been boiled.

Putrificoideae: Clostridiaceae which on meat medium produce after twenty days' incubation under oil at 37° a reaction of pH 7.1 or a more alkaline reaction, the reaction being read after the culture has been boiled.

The name *Clostridioideae* is derived from Prazmowski's generic name *Clostridium*. The name *Putrificoideae* is formed from the specific name *putrificus* (Bienstock 1884). (*Putribacillus vulgaris* of Jensen.) We are hard put to it to find sufficient generic names upon which to form appellations for higher groups in the anaerobic field because *Bacillus* was the generic name applied to any and almost every rod described. But I think that bacteriologists will be justified in using ancient specific names for the formation of the names of tribes and families. Such a proceeding would have a basis in logic if not in precedent. The name *Putrificus* has probably been used for various organisms of the same genus (as well as for those of other genera) and is as much a generic name in sense as though it had been originally designated as such. I should, for example, on finding a slender proteolytic rod that formed terminal oval spores and did not split glucose or other sugars, term it *putrificus type*, knowing that more organisms might be found that corresponded to such a description but that would probably not be specifically identical (see Rodella). Bienstock himself refers to his organism as *B. putrificus* and as *Putrificus* (1899).

A Suggested Classification of the Anaerobic Bacteria

Phylum 1. *Bacteria* (Nov. phyl.): Simple one-celled plants that multiply typically by binary fission and occasionally by budding. They show no form of sexual multiplication. They rarely contain cellulose and do not contain chlorophyll or phycocyanin.

Class 1. *Eubacteriae.*

Class 2. (*Myxobacteriae, Bacteria* which join to form a complex fruiting body (see Vahle, p. 196)).

CLASS 1. *Eubacteriae*: *Bacteria* which do not form a complex fruiting body.

ORDER 1. *Eubacteriales*.

ORDER 2. (*Thiobacteriales*, sulphur bacteria.)

ORDER 3. (*Chlamydobacteriales*, iron and manganese bacteria.)

ORDER 1. *Eubacteriales*: *Eubacteriae* whose cells are never in sheathed filaments. Conidia not observed. Free iron, sulphur, or bacteriopurpurin never present. Multiplication always occurs by transverse fission. (Committee)

FAMILY 6 (?) *Clostridiaceae* (nov. fam.): *Eubacteriales* that are rod-like, not spiral, that will not grow within 7 mm. of the surface of a shaft of clear tissue-free agar medium contained in a tube 12 millimeters or more in diameter, incubated in air, in which they are able to grow in the depths. They may or may not possess peritrichial flagella, they may or may not form endospores. Most members of the group are characterized by their energetic catalytic action on proteins or on carbohydrates or on both of these types of substances.

SUBFAMILY 1. *Clostridioideae* (nov. subfam.): *Clostridiaceae* which on meat medium produce after twenty days' incubation under vaseline at 37° a reaction of pH 7.0 or a more acid reaction, the reaction being read after the culture has been boiled.

Type genus *Rivoltillus* (nov. gen.), the vibrion septique type as described by Heller.

SUBFAMILY 2. *Putrificoideae* (nov. subfam.): *Clostridiaceae* which on meat medium produce after twenty days' incubation at 37° under vaseline a reaction of pH 7.1 or a more alkaline reaction, the reaction being read after the culture has been boiled.

Type genus *Metchnikovillus* (nov. gen.), the sporogenes types as defined in the description of *Bacillus sporogenes*, described by the Medical Research Committee as Metchnikoff's type A.

SUMMARY

1. Morphological criteria cannot be used in classifying the higher groups of anaerobes.

2. The anaerobic rods may logically be placed in a common family on the basis of the physiological character of sensitiveness to free oxygen.

3. This family may be subdivided into two sub-families on the basis of chemical action on carbohydrates and proteins.

4. The divisions "tribe" and "sub-tribe" may well be left open for future organization.

5. The old conception of species in the anaerobic group corresponds to the general systematists' conception of genera. Generic characters may be based chiefly on qualitative behavior on ordinary media. Pathogenesis and general morphology may be used as auxilliary generic characters.

6. Specific characters may be based on sugar fermentations, on quantitative chemical action, on the morphology of colonies and to some extent on the morphology of individuals.

7. We are not as yet ready for extensive biometric determinations in studying many of the anaerobic groups and must temporarily adopt a more easily performed technique for the distinction of these organisms.

8. A classification of the anaerobic group is proposed whose details are to be elaborated in a following paper.

I wish to extend my hearty thanks to Dr. K. F. Meyer and to Dr. Harvey M. Hall for reading the manuscript of this paper and for making many wise and helpful suggestions which have been followed in its compilation.

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HYDROGEN IONS, TITRATION AND THE BUFFER INDEX OF BACTERIOLOGICAL MEDIA

J. HOWARD BROWN

From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey

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Of recent years bacteriologists have become familiar with the determination of hydrogen ion concentration as applied to the problems of bacteriology. In many if not most laboratories media are properly adjusted to certain hydrogen ion concentrations, and the changes in reaction produced by the growth of organisms in these media are likewise determined in terms of hydrogen ion concentration. It has been repeatedly pointed out that media of the same titratable acidity or alkalinity may differ widely in their actual or true acidity or alkalinity. It is known that an acid-forming organism growing well in the presence of an excess of fermentable sugar in different bouillons may arrive at approximately the same final hydrogen ion concentration in each medium, whereas the titratable acidities of the cultures may differ widely. Who of us has not been confronted repeatedly by such questions as—Why do you titrate your cultures? Is not the *true* acidity found by the determination of hydrogen ion concentration and is it not much simpler? Why bother with titration? To which the answer is—Yes, but titration and hydrogen ion determination tell entirely different stories: they are not simply two methods, one more accurate than the other, of determining the same thing.

The committee on the Descriptive Chart of the Society of American Bacteriologists (1919) has published the statement that "the titration method (is) entirely illogical for adjusting the reaction of media or for determining the amount of acid produced by an organism." To both parts of this statement

exception must be taken. The inaccuracies of the titration of media, as it has been commonly practiced in the past, are well known and it is agreed that the reaction of media should be adjusted to certain hydrogen ion concentrations. Nevertheless the committee's condemnation of titration seems entirely too sweeping and therefore misleading.

When one takes a sample of medium and determines how much acid or alkali must be added to bring it to a certain hydrogen ion concentration he performs a titration, though he may choose a better indicator than phenolphthalein and may determine the end point by comparison with a color standard in a comparator block, or may determine the end point electrometrically. If one wishes to determine the reaction of a culture, he must make a hydrogen ion determination, but if he wishes to determine "the amount of acid produced by an organism" he must titrate the culture with a strong alkali, precisely because in a well buffered medium much of the acid formed enters into combination with buffer substances and is not revealed by a hydrogen ion determination.

It has been claimed by H. M. Jones (1920b) that various factors may influence the final hydrogen ion concentration of a culture. Similar conclusions were reached by F. S. Jones (1920) who regards titration under well controlled conditions as quite as satisfactory as the method of determination of hydrogen ion concentration for the study of the fermentative activity of streptococci. The first mentioned author states that "the amount of glucose which a given organism can consume is influenced by the buffer content of the medium . . . which aids in holding the concentration of hydrogen ion from the toxic limit, thus permitting a larger amount of sugar to be decomposed." It follows that in a poorly buffered medium the fermentation of very little glucose is required to raise the acidity to the toxic limit and that therefore in such a medium the presence of a small amount of glucose, as an impurity in a test substance may be a very disturbing factor. The possibility is illustrated by the following experiment.

A culture of *Bact. coli* in Bacto bouillon (poorly buffered) plus 0.1 per cent glucose reached a hydrogen ion concentration of pH 4.8 in 24 hours and remained at this acidity during incubation for 5 days. On the other hand a similar culture in a highly buffered fermented veal infusion bouillon plus 0.1 per cent glucose showed a slight increase in acidity up to 48 hours and then became progressively alkaline reaching a hydrogen ion concentration of pH 8.5 in five days.

It is conceivable that there may be encountered an organism of very low acid tolerance (e.g., pH 6.0 or 6.5) but which may be an active fermenter of various carbohydrates so long as the hydrogen ion concentration is kept down by a well buffered medium. In such a case titration would reveal a considerable amount of acid formed whereas the final hydrogen ion concentration would lead one to believe that little or no fermentation had occurred *unless the buffer content of the medium was well known.*

To be impressed by the importance of a knowledge of the buffer content of media one needs only to note the frequent references to it in the literature, notably the papers by Kligler (1916), Berman and Rettger (1918), Bronfenbrenner and Schlesinger (1918), H. Jones (1920a) and Wolf (1920). Most of the discussion between the protagonists of titration and those of hydrogen ion determination centers about the question of buffer substances. By the use of a color standard of known hydrogen ion concentration and a comparator block the titrationist need no longer be embarrassed by the variable personal equation in judging a poor end point, but to both the titrationist and the hydrogen ion determinist the presence of variable and unknown amounts of buffer substances in media constitutes a real difficulty.

A complete analysis of the reaction between buffer content and growing culture would require a detailed knowledge of the metabolism of the particular organism being cultivated, taking into consideration the fact that the buffer content itself may be modified by the culture. Nevertheless an index of the buffer content at the beginning or at any time during the growth of the culture is readily obtained by titrating the medium with

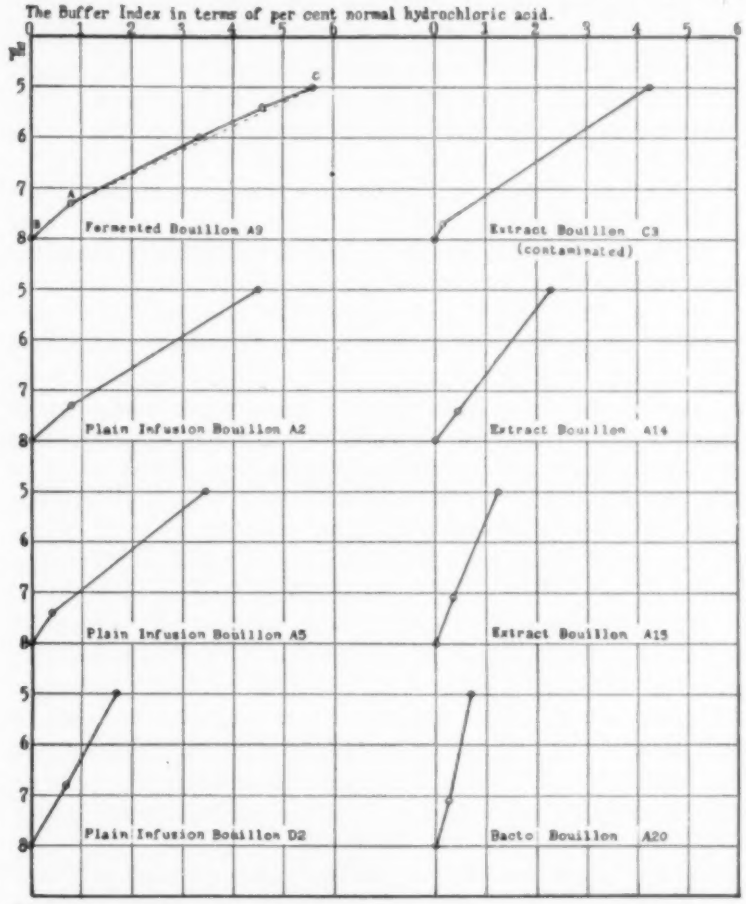
standard acid or alkali from one known hydrogen ion concentration to another. In the determination of such an index two factors must be more or less arbitrarily selected, the limits of hydrogen ion concentration between which titration is to be performed, and the acid or alkali to be used.

Clark (1915b), Bovie (1915), and Clark and Lubs (1917) have published titration curves of bacteriological media. From these curves it is seen that if one starts with a medium of pH 8.0 or a little more alkaline and titrates with hydrochloric acid to pH 5.0 the curve is practically a straight line. If a weak acid such as lactic or acetic acid is used the curve begins to flatten out slightly between pH 6.0 and pH 5.0, and markedly after leaving pH 5.0 because of the lower dissociation constant of the weak acids. The greater part of the range of hydrogen ion concentration of bacteria of interest to the pathologist and sanitarian lies between pH 8.0 and pH 5.0. This is also the range of $Na_2HPO_4 \rightleftharpoons NaH_2PO_4$. The acids formed in cultures are mixtures of weak acids but within the range mentioned the curves of acetic and lactic acid are almost identical with that of hydrochloric acid. It would seem therefore that for the general purpose of determining the relative buffer values of media hydrochloric acid may well be employed.

The "buffer indices" of a number of samples of bouillon from various laboratories have been titrated. The method has been (1) to determine the hydrogen ion concentration of the medium, which usually lies between pH 7.0 and pH 8.0, (2) to add sufficient N/20 NaOH from a burette to reduce the reaction of the sample to pH 8.0, and then (3) to the same or another sample sufficient N/20 HCl to raise the hydrogen ion concentration to pH 5.0. The amount of alkali required to reduce the hydrogen ion concentration of a medium from its initial reaction to a stated lower hydrogen ion concentration, say pH 8.0, may be called the "reserve acidity" (Washburn 1910) of the medium[1]

[1] It is to be noted that the terms "reserve acidity," "reserve alkalinity" and "buffer index" are qualified by the pH values between which the titrations are made. While the determinations here reported are for BI (pH 8–5), for certain problems it may be advisable to adopt other limits of hydrogen ion concentration, as for instance BI (pH 9–5) or BI (pH 5–3).

indicated by the symbols R_H(pH n – 8) in which n = the initial pH. The amount of acid required to raise the hydrogen ion



TEXT-FIG. 1. GRAPHIC REPRESENTATION OF THE BUFFER INDICES OF A NUMBER OF SAMPLES OF BOUILLON

concentration from pH n to pH 5.0 may be called the "reserve alkalinity" (Washburn 1910) of the medium indicated by the symbols R_{OH} (pH n – 5). The "buffer index" indicated by

the symbols BI(pH 8 – 5) is the sum of the reserve acidity plus the reserve alkalinity, each value being expressed in terms of per cent normal acid or alkali, i.e., the number of cubic centimeters of N/1 acid or alkali required to change the hydrogen ion concentration of 100 cc. of medium from one stated hydrogen ion concentration to the other. In figure 1 the reserve acidity is represented on the abscissa by the distance A to B, the reserve alkalinity by A to C, and the buffer index by B to C.[2]

The values referred to may be determined by at least three methods which are as follows. 1. The reserve acidity may be titrated with alkali from pH n to pH 8.0 and then using the same sample the buffer index may be titrated with acid from pH 8.0 to pH 5.0. The reserve alkalinity is calculated by subtracting the former from the latter. 2. The reserve alkalinity may be titrated with acid from pH n to pH 5.0 and then using the same sample the buffer index may be titrated with alkali from pH to 8.0. The reserve acidity is calculated by subtracting the former from the latter. 3. The reserve acidity may be titrated with alkali from pH n to pH 8.0 in one sample and the reserve alkalinity titrated with acid from pH n to pH 5.0 in another sample, the buffer index then being calculated by addition of the other two values. Identical results may be obtained by all three methods if the dilution of the color of the medium and of the indicator is carefully controlled. Many of the results here reported were obtained by the first method. However, the third method is the simplest and is described in detail in the appendix to this paper. It is hardly necessary to point out that the titrations may be controlled electrometrically quite as well as by the colorimetric method, the potentiometer merely taking the place of the color indicators.

Samples of bouillon from five different laboratories, indicated by the letters A, B, C, D, and E, have been titrated and the

[2] Whereas the reserve acidity and reserve alkalinity change with each change in the reaction of the medium, the buffer index may remain constant.

The prevalent method of titrating media against sodium hydrate with phenolphthalein as an indicator is actually a titration of the reserve acidity to an end point of about pH 8.5.

ERRATUM

Journal of Bacteriology, Vol. VI, No. 6, November, 1921. Line 17 from the top of page 560 reads "from pH to 8.0." It should read "from pH 5.0 to 8.0."

TABLE 1

The buffer index of various bouillons

BOUILLON NUMBER	DESCRIPTION	INITIAL pH	BUFFER INDEX pH 8-5	RESERVE ACIDITY pH n-8	RESERVE ALKALINITY pH n-5
A1	Plain veal infusion, 1 per cent Fairchild peptone	7.2	5.25	1.25	4.0
A2	Plain veal infusion, 1 per cent Fairchild peptone	7.3	4.5	0.8	3.7
A3	Plain veal infusion, 1 per cent Fairchild peptone	7.3	4.0	0.75	3.25
A4	Plain veal infusion, 1 per cent Fairchild peptone	7.4	4.0	0.5	3.5
A5	Plain veal infusion, 1 per cent Fairchild peptone	7.4	3.45	0.4	3.05
A6	Plain veal infusion, 1 per cent Fairchild peptone	7.3	3.9	0.75	3.15
A7	Plain veal infusion, 1 per cent Fairchild peptone	7.3	3.85	0.8	3.05
A8a	Plain veal infusion, 1 per cent Fairchild peptone	7.1	3.75	0.95	2.8
A8b	Plain veal infusion, 0.5 per cent aminoids	7.3	3.35	0.55	2.8
A8c	Plain veal infusion, 1 per cent Witte pepton	7.2	3.5	0.6	2.9
A8d	Plain veal infusion, 1 per cent Bacto pepton	7.3	3.45	0.5	2.95
B1	Plain beef infusion bouillon	7.4	5.65	0.75	4.9
B2	Plain beef infusion bouillon	7.6	5.95	0.2	5.75
C1	Plain meat infusion bouillon	7.4	3.6	0.6	3.0
D1	Plain meat infusion bouillon	6.7	3.5	1.7	1.8
D2	Plain meat infusion bouillon	6.8	1.7	0.7	1.0
E1	Plain horse infusion bouillon	6.8	3.5	1.2	2.3
E2	Plain veal infusion bouillon	6.9	3.15	1.2	1.95
A9	Fermented veal infusion bouillon	7.3	5.6	0.75	4.85
A10	Fermented veal infusion bouillon	7.6	5.3	0.6	4.7
A11	Fermented veal infusion bouillon	7.2	5.35	1.0	4.35
A12	Fermented veal infusion bouillon	7.7	5.0	0.15	4.85
C2	Fermented meat infusion bouillon	7.2	4.05	0.75	3.3
D3	Fermented meat infusion bouillon	6.8	3.2	1.1	2.1
E3	Fermented beef infusion bouillon	7.3	3.5	0.5	3.0
A13	0.3 per cent Liebig extract, 1.0 per cent Fairchild pepton	7.4	1.8	0.4	1.4
A14	0.3 per cent Liebig extract, 2.0 per cent Fairchild pepton	7.4	2.3	0.45	1.85
A15	0.3 per cent Liebig extract, 1.0 per cent Witte pepton	7.1	1.25	0.35	0.9

TABLE 1—*Continued*

BOUILLON NUMBER	DESCRIPTION	INITIAL pH	BUFFER INDEX pH 8–5	RESERVE ACIDITY pH 8–5	RESERVE ALKALINITY pH 8–5
A16	0.3 per cent Liebig extract, 1.0 per cent Bacto pepton	7.3	1.25	0.3	.95
A17	0.3 per cent Liebig extract, 1.0 per cent Fairchild pepton	8.1	2.0	−0.15	2.15
A18	0.5 per cent Liebig extract, 1.0 per cent Fairchild pepton	8.0	2.5	0.0	2.5
A19	0.5 per cent Liebig extract, 2.0 per cent Fairchild pepton	8.0	2.9	0.0	2.9
C3	0.5 per cent Liebig extract, 1.0 per cent Bacto pepton (contaminated)	7.7	4.25	0.15	4.1
A20	Bacto bouillon (dehydrated), 0.8 per cent	7.1	0.7	0.25	0.45

The buffer index, reserve acidity and reserve alkalinity are expressed in terms of per cent normal alkali or acid. Note that the buffer index equals the reserve acidity plus the reserve alkalinity.

results tabulated in table 1. Each sample represents a different lot of bouillon. The buffer indices of the samples of plain meat infusion bouillon varied from 1.7 to 5.95. Samples from our own laboratory (A), made at different times but under supposedly uniform conditions, varied from BI 3.45 to 5.25. Of the two samples from laboratory D one had a buffer index of 1.7 and the other 3.5. Four lots of bouillon, A8a, A8b, A8c and A8d were made from the same veal infusion but with different brands of pepton. Their buffer indices were fairly uniform but not very high. Somewhat less variation was shown by the samples of fermented bouillon titrated. Samples of bouillon made from Liebig's beef extract had low buffer indices except sample C3 which titrated BI (pH 8 – 5) = 4.25. This sample was found contaminated by a mixed culture when received. The lowest index was that of sample A20, made of Bacto Nutrient Broth (dehydrated) according to the manufacturer's directions printed on the bottle.

The association of contamination with the high buffer index of extract bouillon C3 suggested that the growth of the culture might have altered the buffer index. Two samples of bouillon

of low buffer index, Bacto bouillon and Liebig extract bouillon, were inoculated with the mixed culture from bouillon C3, incubated forty-eight hours with sterile controls of the same media, and the buffer indices titrated. The results, tabulated in table 2, show that in Bacto bouillon the index was doubled and in extract bouillon was increased slightly. Cultures of *Bact. coli* in the same media showed small increases in the indices of both. A clear centrifugate of the mixed culture in Bacto bouillon had

TABLE 2

The effect of cultures on the buffer index

MEDIUM	CULTURE INCUBATED 48 HOURS	FINAL pH	BUFFER INDEX pH 8-5
Bacto nutrient bouillon	Sterile control	7.2	0.8
	Bact. coli	7.3	0.9
	Mixed culture from extract bouillon C3 (clouded whole culture)	6.8	1.6
	Clear centrifugate from the above	6.9	1.5
Beef extract bouillon	Sterile control	7.4	1.55
	Bact. coli	7.5	2.05
	Mixed culture from extract bouillon C3	7.4	1.6
Fermented bouillon	Sterile control	7.6	4.25
	Bact. alkaligenes	8.4	5.1
Plain veal bouillon	Sterile control	7.4	4.0
	Bact. alkaligenes	8.1	4.2

practically the same buffer index as the clouded culture, showing that the increase was not due to the presence of bacterial bodies but to substances in solution. A similar experiment was conducted with *Bact. alkaligenes* using fermented bouillon and plain unfermented veal infusion bouillon. There was an appreciable increase in the buffer index of the culture in fermented bouillon but only a slight increase in that of the plain bouillon. In other experiments the buffer indices of cultures of *Bact. coli* in plain veal bouillon increased markedly, but when an excess of glucose was added little or no change in the buffer index

occurred. A hemolytic streptococcus produced no change in the buffer index of glucose bouillon though the hydrogen ion concentration increased from pH 7.1 to pH 4.7 during incubation for eight days. The results of these experiments suggest that in the cases mentioned the increase in buffer index was the result of protein metabolism. It is suggested that the ability or failure of a culture to produce changes in the buffer indices of media may be of differential value.

TABLE 3

Influence of the reserve alkalinity and the amount of fermentable sugar on the final hydrogen ion concentration

MEDIUM: VEAL INFUSION BOUILLON				CULTURE OF BACT. COLI		
Dextrose per cent	Initial pH	BI (pH 8-5)	Ron (pH n-5)	48 hours pH	96 hours pH	144 hours pH
0.5	6.2	4.45	1.95	5.1	5.8	7.5
0.5	7.5	4.1	3.6	5.5	6.5	7.8
0.75	6.2	4.4	2.0	5.1	5.1	5.4
0.75	7.5	4.2	3.6	5.2	5.5	7.0
1.0	6.2	4.3	1.9	5.1	5.0	4.9
1.0	7.4	4.2	3.5	5.1	5.2	5.3
1.25	6.1	4.2	1.65	5.1	5.0	4.9
1.25	7.4	4.4	3.7	5.1	5.1	5.1

In figure 1 are plotted the buffer indices of a number of bouillons. The pH values are located along the ordinate axis and the percentage of normal acid or alkali used on the abscissa. By the simple methods of titration described above three points are located, the initial pH at A (as shown on the curve of fermented bouillon A9), pH 8.0 at B, and pH 5.0 at C. If other points between A and C are determined they are found to lie very close to the straight line from A to C. The true form of the curve for fermented bouillon A9 is shown. A comparison of the curves shown in figure 1 shows that the smaller the buffer index the more nearly does the curve approach a straight line.

In a certain lot of bouillon containing 1 per cent of glucose *Bact. coli* produced an alkaline reaction after incubation for four days. This bouillon had a high buffer index and reserve alkalinity. The experiment recorded in table 3 was designed to explain this phenomenon and shows that the reserve alkalinity of a medium may be of diagnostic importance. It is seen that in a bouillon with a reserve alkalinity (pH n – 5) of 3.5 per cent normal, 1 per cent of glucose was hardly sufficient to insure continued acidity. The culture in the same medium containing 0.75 per cent of glucose actually became alkaline to brom cresol purple (pH 7.0) in one hundred forty-four hours. On

TABLE 4

Acid production by Bacterium coli

MEDIUM			CHANGES DURING INCUBATION				
			6 hours		25 hours		
Composition	pH	BI (pH 8-5)	Turbidity	pH	Turbidity	pH	Titration to pH 8
Bacto bouillon + 1 per cent glucose	7.0	0.7	++	5.0	++	4.8	1.25
Veal bouillon + 1 per cent glucose	6.9	5.2	+++	6.1	++++	5.5	4.85

The titration is expressed in terms of per cent normal acid or cubic centimeters of N /20 NaOH required to reduce the acidity of 5 cc. of culture to pH 8.0.

the other hand if the reserve alkalinity was reduced to about 2.0, less than 1 per cent of glucose was sufficient to maintain the acidity of the culture.

Of what value would the buffer indices illustrated in figure 1 be to the bacteriologist in selecting his medium? To mention only one or two illustrations; if he were working with a member of the *Bacterium coli* group and wished to determine in a few hours whether the organism would ferment sucrose, he might select a bouillon with a low buffer index, i.e., one in which the formation of a small amount of acid would be revealed by a rapidly rising hydrogen ion concentration. If, on the other hand, he desired abundant growth and the production of a

large amount of acid he would do well to select a medium with a high buffer index and high reserve alkalinity. This is illustrated in the experiment recorded in table 4, in which *Bact. coli* was grown in glucose bouillons of low and high buffer indices. The *degree* of acidity rose much more quickly and the final hydrogen ion concentration was higher in the bouillon of low buffer index but the *amount* of acid produced was much greater in the bouillon of high buffer index. The experiments recorded in tables 4 and 5 also illustrate the statement of Clark (1915a) that "unless the media employed by different laboratories are identical, at least in their buffer effect, the titratable acidity

TABLE 5

Acid production by a streptococcus

MEDIUM			CULTURES INCUBATED 8 DAYS	
Composition	pH	BI (pH 8-5)	Final pH	Titration to pH 8.0
Veal bouillon + 1.0 per cent glucose, Lot 1	7.1	3.75	4.7	3.9
Veal bouillon + 1.0 per cent glucose, Lot 2	6.9	5.2	4.9	5.3

The titration is expressed in terms of per cent normal acid or cubic centimeters of N /20 NaOH required to reduce 5 cc. of culture to pH 8.0.

produced by the same organism may be found to be very different." They also confirm his observation that "the greater the buffer effect of the medium, the lower the final hydrogen ion concentration attained." It may be added, therefore, that if it is desired to compare the final hydrogen ion concentrations or the titratable acidities of similar cultures in different media, at least the buffer indices of the media should be known. It is not claimed that the titratable acidity is always a measure of the amount of acid produced, nor that the buffer content is the only factor which determines the amount of acid which may be produced or the amount of growth which a medium can support. There may be simultaneous production of acid and alkali by some organisms. It has been shown above that the buffer content may be altered by the growth of the culture.

Many organisms grow less abundantly in fermented bouillon plus glucose than in unfermented bouillon plus glucose although the two media have equally high buffer indices. Nevertheless, the buffer index is one of the most important factors and one which should be determined.[3]

CONCLUSIONS

The titration of media is not to be regarded as a crude method of determining the reaction of media but a process which reveals facts not disclosed by a simple hydrogen ion determination.

For many common purposes a knowledge of the buffer content of media is quite as important as the hydrogen ion concentration.

The buffer content between stated limits of hydrogen ion concentration is easily defined as the *buffer index* which is the sum of the *reserve acidity* and *reserve alkalinity* between those limits.

A simple colorimetric method of determining these values is described, a method which need not consume more than five minutes time. The determination can be made by any laboratory helper who can make a titration or a hydrogen ion determination and should be recorded for each lot of medium made.

There is appended a copy of instructions for laboratory helpers and a convenient form of record on which is recorded a sample titration.

The author wishes to acknowledge his indebtedness to Dr. P. E. Howe of this department for valuable suggestions and criticisms.

[3] Since this paper has been written there has appeared the paper on The Relation of Hydrogen-ion Concentration to the Growth, Viability, and Fermentative Activity of Streptococcus hemolyticus by L. F. Foster (Jour. Bact., March, 1921, **6**, 161). The author illustrates admirably some of the points brought out in the present paper. He emphasizes the necessity of knowing the buffer content of a medium.

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APPENDIX

METHOD FOR THE TITRATION OF MEDIA

Equipment

A set of colorimetric hydrogen ion standards of the following ranges and containing the indicators mentioned.

pH 5.0 – 5.8 (methyl red)
pH 5.8 – 6.8 (brom cresol purple)
pH 6.8 – 8.0 (phenol red)

A comparator block.

Drop bottles or pipette bottles containing the indicators mentioned.

Solutions of N/20 NaOH and N/20 HCl.

Two finely graduated burettes.

A very accurate 1 cc. or graduated 2 cc. pipette.

Tubes of uniform internal diameter similar to those containing the standards. Ordinary potato tubes of NONSOL glass are satisfactory.

Distilled water.

Method

1. Into each of 3 clean tubes place 9 cc. of distilled water and 1 cc. (very accurately measured) of the medium to be titrated.

2. Make the hydrogen ion determination in the usual manner, using one of the tubes (*tube I*) as a color screen and adding phenol red to another (*tube II*). (If the hydrogen ion concentration of the medium lies outside the color range of phenol red but inside the range of brom cresol purple (pH 5.8 to 6.8) the pH determination had better be made in a separate sample which is then discarded.)

Record the hydrogen ion concentration on the record sheet.

3. Place the pH 8.0 standard in the comparator block behind the color screen (*tube I*). From the burette cautiously add N/20 NaOH to *tube II* (containing phenol red) until its hydrogen ion concentration becomes pH 8.0 as determined by viewing it in the comparator block beside *tube I*.

Record figures in spaces a, b, and c of the record sheet.

4. Place the pH 5.0 standard in the comparator block behind *tube I*. Discard *tube II* and to a third tube of the diluted medium (*tube III*) add methyl red. From a burette cautiously add N/20 HCl to *tube III* until its hydrogen ion concentration becomes pH 5.0.

Record figures in spaces e, f, and g of the record sheet.

5. Calculate the reserve acidity and reserve alkalinity as indicated on the record sheet. The buffer index is the sum of the reserve acidity plus the reserve alkalinity.

Record these values on the record sheet.

Notes

Greater accuracy is attained by using N/20 NaOH and N/20 HCl containing phenol red and methyl red respectively in the same concentration as is present in the color standards, as suggested by Hurowitz, Meyer and Ostenberg (1915).

If sufficient volumes of N/20 NaOH or HCl are added to tubes II or III to change the volume of the contents appreciably, the volume of the contents of the color screen, tube I, should be increased equally by the addition of distilled water or of N/20 alkali or acid without indicator.

Determinations should be made in duplicate, at least until the technician has perfect confidence in his results.

Record of titration of medium

Medium *Fermented Veal Infusion Bouillon No. A9*
Sample (x) = 1 cc. of medium in each tube.
Initial hydrogen ion concentration (n) = pH 7.3.
Titration of reserve acidity with N/20 NaOH from initial pH 7.3 to pH 8.0

		Sample I	*Sample II*
Burette reading	(a)	0.00	0.15
Burette reading	(b)	0.15	0.3
Difference	(c)	0.15	0.15
Average	(d)	0.15	

Reserve acidity = d × 5 ÷ x = 0.75

Titration of reserve alkalinity with N/20 HCl from initial pH 7.3 to pH 5.0

		Sample I	*Sample II*
Burette reading	(e)	1.32	2.28
Burette reading	(f)	2.28	3.26
Difference	(g)	0.96	0.98
Average	(h)	0.97	

Reserve alkalinity = h × 5 ÷ x = 4.85

Calculation of buffer index;

Reserve acidity, R_H (pH n — 8)	= 0.75
Reserve alkalinity, R_{OH} (pH n — 5)	= 4.85
Buffer index, BI (pH 8 — 5)	= 5.60

ON DECREASING THE EXPOSURE NECESSARY FOR THE GELATIN DETERMINATION

J. E. RUSH AND G. A. PALMER

Sanitary Engineering Department, Carnegie Institute of Technology, Pittsburgh, Pennsylvania

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Some time ago, one of us (J. E. R.) was advised of the fact that there was a more rapid method for determining the ability of organisms to liquefy gelatin than the routine one, in use in most laboratories, namely, subjecting the gelatin stab to a temperature of 20°C. for ten or fourteen days after inoculation and then noting the results. Search of the literature failed to reveal any description of the more rapid method which was described as incubation at 37°C. for four days followed by twenty-four hours incubation at 20°C. after which the results were recorded.

Many reasons recommend the latter procedure providing the results obtained are identical with those recorded by the present recognized procedure. Among such reasons we might enumerate:

1. The saving of time (five days requirement as contrasted with fourteen days).
2. Earlier liberation of test tubes from the incubator (which in a busy laboratory is an important factor).
3. Necessity of less incubator space to meet the requirements of any laboratory (in some cases dispensing altogether with the use of a 20°C. incubator).

As search of the literature failed to reveal any information on this point it was determined to test out the two methods simultaneously on the next set of cultures which came into the laboratory and upon which confirmatory work (including the gelatin reaction) was to be done. This opportunity came when

it was desired to examine several organisms isolated from a water supply and upon which confirmatory work for *Bact. coli* was necessary.

Inoculations of the same batch of gelatin were made in duplicate and controls from the same batch of media were used. In the making of the media the standard procedure[1] was used, i.e., 10 per cent gelatin was made and the final reaction was adjusted to + 1 on the phenolphthalein scale. As above stated inoculations were made in duplicate of the cultures to be examined—one set was placed in the 20°C. incubator, following the generally accepted method of procedure and observations were made after ten and fourteen days (as recorded below). The other set was placed at 37°C. for four days and then transferred to the 20°C. incubator for twenty-four hours after which the results were recorded.

It is a well recognized fact, that in a number of gelatin stabs exposed to 20°C. the longer the time of exposure, the greater will be the percentage showing liquefaction. This has been definitely shown by Gage and Phelps[2] but so far as we are aware the relation between a certain time (ten to fourteen days) at 20°C. and the combination incubation, first at 37°C. and later at 20°C. has not been determined. Prolonged high temperature will keep gelatin from solidifying again but this did not obtain here with exposure of the gelatin to 37°C. for four days, as evidenced by the fact that the controls on the media, while liquid after removal from the 37°C. incubator were again solid when removed from the 20°C. incubator after a further exposure of twenty-four hours.

This gelatin inoculation is made, as is well recognized, for the simple purpose of determining the presence or absence of certain proteolytic enzymes, namely, gelatinases. As in other biochemical reactions, the rate of reaction is influenced by certain factors in the environment and one of the most important factors is that of temperature. The cultures experimented

[1] Standard Methods for Examination of Water and Sewage, 1920.

[2] Gage and Phelps. Quoted by Prescott and Winslow. Elements of Water Bacteriology. John Wiley and Sons. 1915.

with, probably represent an average population of those organisms found in water supplies and giving a positive presumptive test for *Bacț. coli*, because they are from many sources and sampled under a variety of conditions.

It should be further recalled that the optimum temperature of these organisms, undoubtedly varied considerably and that they probably exhibited varying degrees of adaptation. In addition, it may well be assumed that the difference between exposure at 37°C. and 20°C. even for a short time would be sufficient to stimulate a gelatinase production in certain forms while inhibiting it in others. That we are probably not dealing with a single enzyme which has the power of gelatin liquefaction, and that our problem is thus complicated is evident from certain work on zymology.[3]

The general results of our tests are shown in the table below:

LIQUEFACTION AFTER		NUMBER STRAINS
Four days at 37° and one day at 20°	Fourteen days at 20°	
None	None	97
None	Slight	78
None	Marked	17
Slight	Slight	8
Marked	Slight	4
Complete	None	1
Complete	Slight	8
Complete	Marked	8

"Slight" signifies 20 per cent liquefaction or less; "Marked" signifies 25 per cent liquefaction or more.

CONCLUSIONS

From the table it will be seen that no very definite statements can be made except that if we desire to get the results (as ordinarily done now) by exposure to 20°C. for ten or fourteen days—these same results cannot be realized by exposure to 37°C. for four days and then to 20°C. for one day.

[3] Biochemical Catalysts in Life and Industry. Effront and Prescott. John Wiley and Sons. 1917.

It was naturally noted (as previously reported) that a greater number of inoculations show liquefaction at 20°C. as time progresses (comparing results at 20°C. for ten days with those at the same temperature for fourteen days). It should also be noted that certain cases show a definite increase in percentage liquefaction from the ten to the fourteen day period. The number of tubes which show liquefaction at fourteen days and none at ten days is however less than one-half of one per cent of the total cultures examined.

As a general thing more cultures show liquefaction, or there is an increased amount of liquefaction, at 20°C. for fourteen days than by the method of exposure to 37°C. for four days and to 20°C. for one day.

In those inoculations in which a greater liquefaction by the more rapid method was noted, it may be pointed out that in practically every case total liquefaction occurred. Whether or not this has any significance we are unable to say.

CHART OF THE FAMILIES AND GENERA OF THE BACTERIA

HAROLD MACY

From the Dairy Bacteriology Laboratory, University of Minnesota

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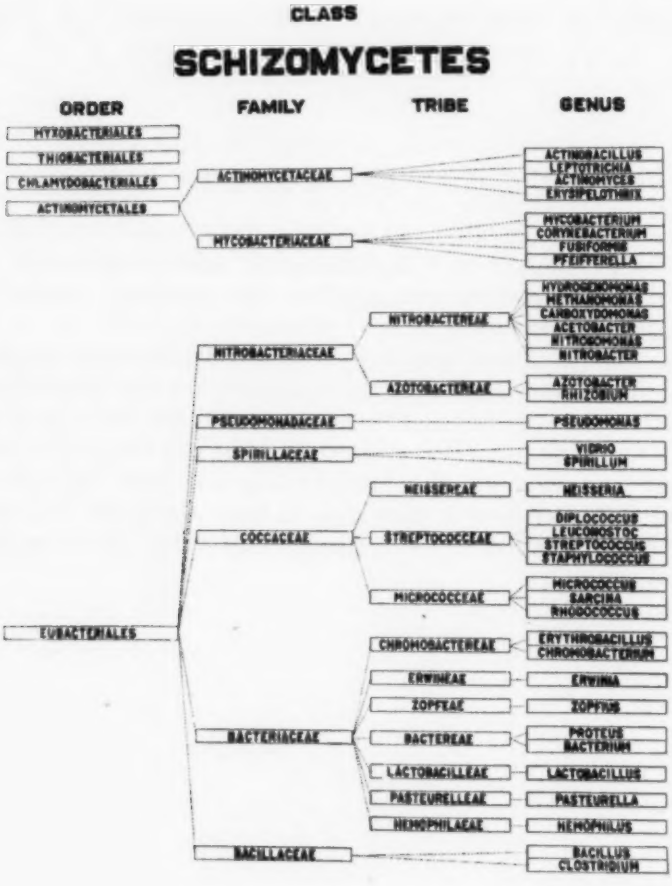
The final report[1] of the Committee of the Society of American Bacteriologists on Characterization and Classification of Bacterial Types offers a suggestive and tentative outline of bacterial classification.

The idea of preparing a chart which would illustrate graphically the position of the orders, families, tribes and genera presented itself to the writer, with the result that the accompanying chart was prepared. It is realized that the classification is not, in any way, final but it is thought that the chart may prove useful to bacteriologists who wish to have a convenient guide to the arrangement of the Schizomycetes under the proposed classification.

[1] C.-E. A. Winslow, Chairman, Jean Broadhurst, R. E. Buchanan, Charles Krumwiede, Jr., L. A. Rogers, and G. H. Smith. The Families and Genera of the Bacteria. Jour. Bact., vol. V, no. 3, May, 1920, pp. 191–215.


CLASS
SCHIZOMYCETES
ORDER
FAMILY
TRIBE
GENUS
MYXOBACTERIALES
THIOBACTERIALES
CHLAMYDOBACTERIALES
ACTINOMYCETALES
ACTINOMYCETACEAE
MYCOBACTERIACEAE
ACTINOBACILLUS
LEPTOTRICHIA
ACTINOMYCES
ERYSIPELOTHRIX
MYCOBACTERIUM
CORYNEBACTERIUM
FUSIFORMIS
PFEIFFERELLA
EUBACTERIALES
NITROBACTERIACEAE
PSEUDOMONADACEAE
SPIRILLACEAE
COCCACEAE
BACTERIACEAE
BACILLACEAE
NITROBACTEREAE
AZOTOBACTEREAE
NEISSEREAE
STREPTOCOCCEAE
MICROCOCCEAE
CHROMOBACTEREAE
ERWINEAE
ZOPFEAE
BACTEREAE
LACTOBACILLEAE
PASTEURELLEAE
HEMOPHILACEAE
HYDROGENOMONAS
METHANOMONAS
CARBOXYDOMONAS
ACETOBACTER
NITROSOMONAS
NITROBACTER
AZOTOBACTER
RHIZOBIUM
PSEUDOMONAS
VIBRIO
SPIRILLUM
NEISSERIA
DIPLOCOCCUS
LEUCONOSTOC
STREPTOCOCCUS
STAPHYLOCOCCUS
MICROCOCCUS
SARCINA
RHODOCOCCUS
ERYTHROBACILLUS
CHROMOBACTERIUM
ERWINIA
ZOPFIUS
PROTEUS
BACTERIUM
LACTOBACILLUS
PASTEURELLA
HEMOPHILUS
BACILLUS
CLOSTRIDIUM

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Per foot (see Special Notice)						

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Per foot (see Special Notice)						

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Thickness of wall, inches	1/16	1/16	1/16	1/16	1/16	1/16
Approximate no. feet per box	60	60	15	15	14	10
Per foot (see Special Notice)						

11665. RUBBER TUBING, Red, Antimony, heavy wall, same quality as No. 11660. Preferred by many users for Bunsen burner tubing in place of No. 11655.

Diameter inside, inches	1/8	1/4	3/8	1/2	3/4	1
Thickness of wall, inches	1/16	1/16	1/16	1/16	1/16	1/16
Approximate no. feet per box	12	12	10	10	10	10
Per foot (see Special Notice)						

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Table Showing Approximate Number of Rubber Stoppers in One Pound

Number	00	0	1	2	3	4	5	6	7	8	9	10	11	12	13
Diameter large end, mm.	14	17	19	21	23	25	27	29	31	33	35	37	39	41	43
Diameter small end, mm.	9	12	13	15	17	19	21	23	25	27	29	31	33	35	37
Approximate Solid	1000	600	400	300	200	150	100	75	60	50	40	30	25	20	15
Number in One Pound 1 hole	800	500	350	250	175	125	90	70	55	45	35	28	23	18	14
2 holes	700	450	300	225	150	110	80	60	50	40	30	25	20	16	12

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